An *in vitro* study of the susceptibilities and growth dynamics of common ocular pathogens using five fluoroquinolones

A Thesis Submitted to the

College of Graduate Studies and Research

in partial fulfillment of the requirements for the

Master of Science Degree in the

Department of Microbiology and Immunology

University of Saskatchewan

Saskatoon

By

Peter D. S. Hedlin

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of the university may make it freely available for inspection. I further agree that permission to copy this thesis in any way, for scholarly purposes only, may be granted by the professor or professors who supervised my thesis work, or in their absence, by the Head of the Department of Microbiology and Immunology or the Dean of the College of Graduate Studies and Research. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without written permission. It is also understood that due recognition shall be given to me and the University of Saskatchewan in any scholarly use which may be made of any materials in my thesis.

All requests for permission to copy or to make use of material in this thesis in part or whole should be addressed to:

Head of the Department of Microbiology and Immunology
University of Saskatchewan
Saskatoon, Saskatchewan
S7N 5E5

ABSTRACT

Bacteria are responsible for up to 70% of all ocular infections including conjunctivitis, keratitis and endophthalmitis. If left untreated, a reduction of visual acuity, and in severe cases, sight loss, is possible. Treatment usually consists of a topically applied antibacterial preparation for patients with superficial infections. With intraocular infections, topical administration is augmented with systemic treatment or local instillation. While several types of drugs are available for ocular therapy, the fluoroquinolone class of antimicrobials is especially effective. This is due in part to their broad-spectrum of activity and low toxicity. However, as with any globally prescribed antimicrobial agent, bacterial resistance is an issue. Over the past 10 years there has been a decline in the effectiveness of older fluoroquinolones (ciprofloxacin and ofloxacin) in treating Gram-positive and, to a lesser extent, certain Gram-negative infections. In response to the declining activity of ciprofloxacin and ofloxacin, newer fluoroquinolones have been developed such as levofloxacin (L-isomer of ofloxacin), and more recently, gatifloxacin and moxifloxacin.

In order to ensure the most potent drugs are being used to treat the most serious types of infection, studies need to be done to assess the activity of the current antimicrobial arsenal against pertinent infecting organisms. Three different types of experiments can be done to achieve this. *In vitro* potency can be tested two ways. The first is minimum inhibitory concentration (MIC). This test defines the concentration of antimicrobial drug that prevents growth of bacteria when tested against an inoculum of approximately 10^5 colony forming units (CFU)/ml. The second is the mutant prevention concentration (MPC), which is the amount of drug needed to inhibit a first step resistant mutant. This is a relatively new approach to measuring fluoroquinolone potency; like MIC it is not a measure of kill. A separate set of experiments are needed to assess *in vitro* killing. Kill curves measure the ability of an antimicrobial agent to reduce/kill a bacterial population over a period of 24 hours.

Because bacterial loads can vary greatly in *in vivo* infections, kill curves were conducted on a series of four inoculum sizes ranging from 10^6 to 10^9 cfu/ml. Some of the most common ocular pathogens are *Streptococcus pneumoniae*, *Staphylococcus aureus*,

Haemophilus influenzae and *Pseudomonas aeruginosa*. *Mycobacterium fortuitum* and *Mycobacterium chelonae*, while much less commonly associated with ocular disease, are capable of causing vision-threatening infections. As a result, the above six organisms were used to test the *in vitro* potency of ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin and gatifloxacin.

Both MIC and MPC testing found both gatifloxacin and moxifloxacin to be 4-8-fold more potent *in vitro* against the Gram-positive organisms than the older fluoroquinolones with an average potency rank order of moxifloxacin = gatifloxacin > levofloxacin > ofloxacin = ciprofloxacin. The Gram-negative results, however, revealed that the older fluoroquinolones are still the most potent of the fluoroquinolones tested with an average potency rank order of ciprofloxacin > ofloxacin = levofloxacin > gatifloxacin = moxifloxacin.

Kill curve results showed a significant difference in the rate of killing between the MIC and MPC drug concentrations. At the MIC drug concentration there was generally only a noticeable reduction in viable cells following 24 hours of drug exposure and in many cases this was followed by a period of bacterial re-growth. At the MPC drug concentration, a significant bacterial count reduction was often observed as early as 4 to 6 hours for both *S. pneumoniae* and *H. influenzae*. Surprisingly, there was little difference between the five fluoroquinolones in their rates of and amount of bacterial reduction.

Because of high *in vitro* resistance rates in drugs like penicillin, the fluoroquinolones are an important broad-spectrum alternative. Consequently, it is imperative that measures are taken to maintain the efficacy of this class. One approach is to ensure that the most potent drug is being used to eradicate possible resistant sub-populations present in *in vivo* infections. The data from these experiments suggest that the new fluoroquinolones gatifloxacin and moxifloxacin are much more potent (*in vitro*) than older fluoroquinolones against Gram-positive bacteria. With Gram-negative pathogens, however, ciprofloxacin remains the most potent agent *in vitro*.

ACKNOWLEDGEMENTS

The completion of this project would not have been possible had it not been for the kindness, knowledge, generosity, resources and support of a great number of people and a few key organizations.

I would like to express my gratitude to Dr. Joseph Blondeau for providing me with the opportunity to pursue a graduate project in an area I am truly fascinated with. Your experience and knowledge and willingness to help have been a greatly appreciated.

I would like to thank the members of my graduate committee Dr. Deneer, Dr. Sanche and Dr. Qualtiere whose guidance throughout my project was invaluable. They were so easy to work and were extremely supportive throughout the entire process. I would also like to thank them for kindly providing letters of reference for my future endeavors.

I would also like to say thank you to those who I worked with on a regular basis. Shantelle you are truly a wizard of the lab. I would have been lost with out you. Your impeccable organization, willingness to help and bubbly personality made my time there extremely enjoyable. I would also like to thank Kelli and Chris for being terrific class/lab mates and friends for the last many years, and to Glen for his advice along the way. Mary and Dawn have also been a great help and resource. I would also like to give a shout out to the entire RUH Micro lab group and wash-up staff. You guys made me laugh every single day.

Thanks also to Allergan and the Department of Microbiology and Immunology at the University of Saskatchewan for providing financial support for research and travel to the ARVO conference in Florida.

Finally, I would like to thank my family, mom and dad, Matt and Erik and my grandparents for their unconditional support and encouragement, as well as their fabulous home-cooked meals and intense audible squash games. Thank you Cherise for all of those movie nights we've had (I think we've seen everything in Blockbusters' inventory) and for being my best friend through all of this.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
ATTC	American Type Culture and Collection
AUC	Area Under the Curve
AUIC	Area Under the Inhibitory Curve
cfu	Colony Forming Units
cipro	Ciprofloxacin
FQ	Fluoroquinolone
gati	Gatifloxacin
HTM	Haemophilus Test Media
levo	Levofloxacin
L	Litre
MHB	Mueller Hinton Broth
MSSA	Methicillin Susceptible *Staphylococcus aureus*
μg	Microgram
μl	Microlitre
mg	Milligram
ml	Millilitre
MIC	Minimum Inhibitory Concentration
M	Molar
moxi	Moxifloxacin
MPC	Mutant Prevention Concentration
nm	nanometer
NaOH	Sodium Hydroxide
NAD	β-Nicotinamide Adenine Dinucleotide
CLSI	Clinical and Laboratory Standards Institute
N	Normality
oflox	Ofloxacin
O.D.	Optical Density
PK/PD	Pharmacokinetic/Pharmacodynamic
rpm	Rotations Per Minute
THB	Todd Hewitt Broth
TSA	Tryptic Soy Agar

1.0 Introduction

1.1 General Overview

Ocular infections account for 1-4% of all patient-physician visits and result primarily from bacterial or viral pathogens (1). Typically the causative bacterial organisms are *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*. While there are a wide variety of other organisms that also have the potential to cause infection, the genus and species listed above are the most common. The ocular structures most often involved in an infection are the conjunctiva, cornea and aqueous/vitreous humor.

1.2 Ocular Physiology

1.2.1 Conjunctiva

The conjunctiva consists of a mucous membrane that lines the inner portions of the eyelids and is reflected onto the globe covering the sclera to the limbus. It is made up of three sections: the palpebral/tarsal conjunctiva, fornix conjunctiva and the bulbar conjunctiva (Fig 1.1)(2).

The conjunctiva possesses several mechanisms of defense which can be categorized as non-immune and immune. The non-immune mechanisms are barriers which help protect the underlying conjunctival tissues from infection. These include the intact mucous membrane surface, rapid epithelial cell turnover, cool temperature due to tear evaporation, the mechanical action of the eyelids and the flushing action of the tears and lacrimal system (2). The normal flora (*Staphylococcus epidermidis*, *S. aureus* and *Corynebacterium* species) are typically non-virulent organisms which colonize the ocular surfaces and metabolize the nutrients available in the ocular environment. This makes it difficult for more virulent organisms (which may be of the same species as the normal flora) to proliferate in the same area causing an infection. In addition, the tear film constituents lactoferrin, β-lysine and lysozyme have antibacterial actions which complement the anatomical barriers (3). Often during an infection, a dry eye state occurs due to a diminished tear film. To compensate for this, additional antibacterial proteins may leak out from inflamed blood vessels (4).

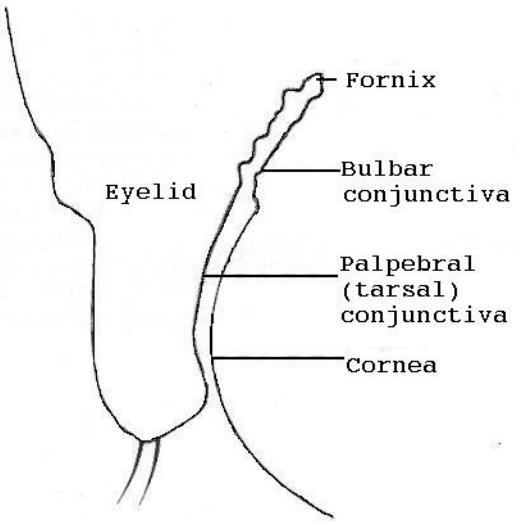

Figure 1.1: Anatomic division of the conjunctiva (5).

The conjunctiva is a highly vascularized structure. As a result, all cellular components of the immune system are present in the conjunctival substantia propria except basophils and eosinophils (2).

While the tear film is technically a separate entity, its structure and function is so closely linked to that of the conjunctiva that it will be discussed in the same section. The tear film is a three layered structure consisting of an outer lipid layer, a middle aqueous layer which is approximately 7-10 mm thick and a mucous layer (6). Each of the three layers is secreted by different tissues. The outer lipid layer is secreted primarily by meibomian glands which are located within the lower and upper eyelids. The constituents of this layer include wax monoesters, sterol esters, hydrocarbons, triglycerides, diglycerides, free sterols, free fatty acids and polar lipids (7). The middle aqueous layer is secreted by the main lacrimal gland, accessory lacrimal gland and both the corneal and conjunctival epithelial cells. This layer is made up of antibacterial proteins, immunoglobins, growth factors, lysozyme, lactoferrin, secretory IgA, electrolytes and water (8). The inner mucous layer is produced by conjunctival goblet cells as well as stratified squamous cell from the conjunctiva and cornea (Fig 1.2)(9).

The tear film is essential for the health, maintenance, and protection of the ocular surface. It contributes to the smooth optical properties of the corneal surface. It is a primary source of oxygen to the cornea. It acts as a lubricant between the eye lids and ocular surface. Also, the movement of tears across the ocular surface and their drainage into the nasolacrimal duct help remove foreign bodies, debris and exfoliated cells. Finally, as mentioned above, the tear film contains antibacterial proteins which help protect the conjunctiva and cornea (9)

1.2.2 Cornea

The cornea is a unique assembly of structures that when combined produce an almost perfectly transparent, avascular optical tissue that serves as a physical barrier between the environment and the inside of the eye. It is also a major refractive structure that focuses light onto the retina (9). The cornea is composed of six layers which vary greatly in thickness. The outermost layer is the epithelium. It is 5-7 cells thick and is made up of three types of cells: basal cells, wing cells and superficial cells. The basal cells form a single cell layer which is attached to the basement membrane via

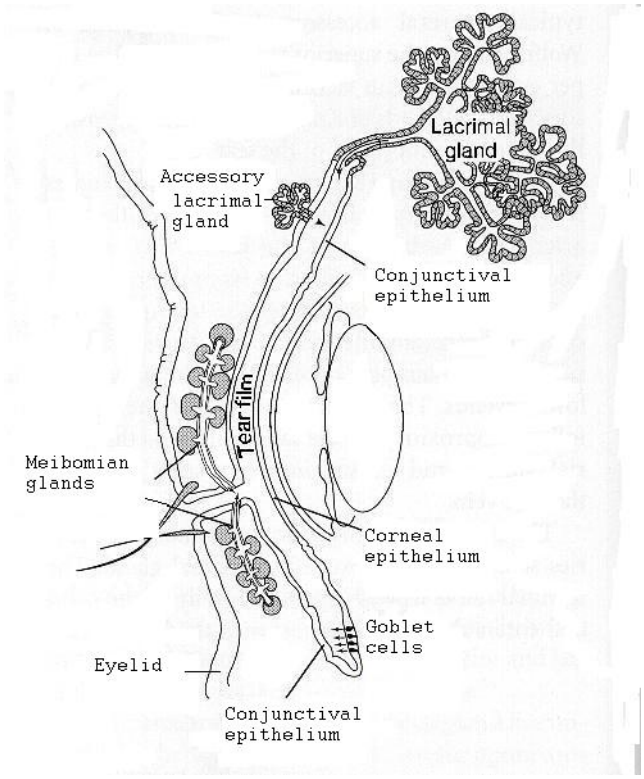

Figure 1.2: Schematic showing the glands and epithelia of the eye and ocular surface that contribute to the tear film. (9)

hemidesmosomes. It is at this layer where mitosis occurs. As cell division occurs the cells begin to differentiate, forming the wing cells which are one to three layers thick. These cells represent an intermediate state of differentiation. The superficial cells are terminally differentiated and make up the outermost layer which is three to four cells thick. At this point the cells degenerate and are sloughed off from the corneal surface. The epithelium has a turnover rate of about seven days (10). As a result, a corneal injury that damages only the epithelium will heal without a scar.

The second layer is only 40-60 μm thick and is made up of type IV collagen, type VII collagen, laminin, heparin sulfate and proteoglycan (11). The third layer is the Bowman's layer. It is 12 μm thick and is made up of randomly arranged type I collagen fibers. It is thought that this layer acts as a stabilizing element in the cornea (12).

The stroma is the fourth and thickest layer. Making up 90% of the thickness of the cornea, it is 0.65 mm thick at the peripheral portions and 0.52 mm at the central portion (13). The Descemet's membrane is the fifth layer. Its thickness is 10-15 μm early on in life, and increases in thickness with age. This membrane in secreted by the endothelial cells and is made up type IV collagen, laminin and fibronectin. The fibronectin is responsible for the adhesion of this membrane to the endothelial cells (14).

The endothial cells make up the final and innermost layer of the cornea (Fig 1.3) (9). It is a single layer of hexagonal cells (4-6 μm thick) which are incapable of replicating. With age, the number of endothial cells decreases. As a result, the neighboring cells enlarge to fill the gap (15). The junctions between cells are such that a leaky barrier is formed between the aqueous humor and the stroma. This allows the entry of nutrients into the avascular stroma. There is also an endothelial system of metabolic pumps, ion transporters and channels which interact to osmotically remove water from the stroma to ensure corneal transparency (16).

1.2.3 Aqueous Humor

The aqueous humor is a liquid body located immediately behind the cornea, just in front of the lens. It was previously thought that this was a stagnant fluid, but it has since been shown to be continuously formed and drained (17). Three physiological processes are involved in the formation and chemical composition of the

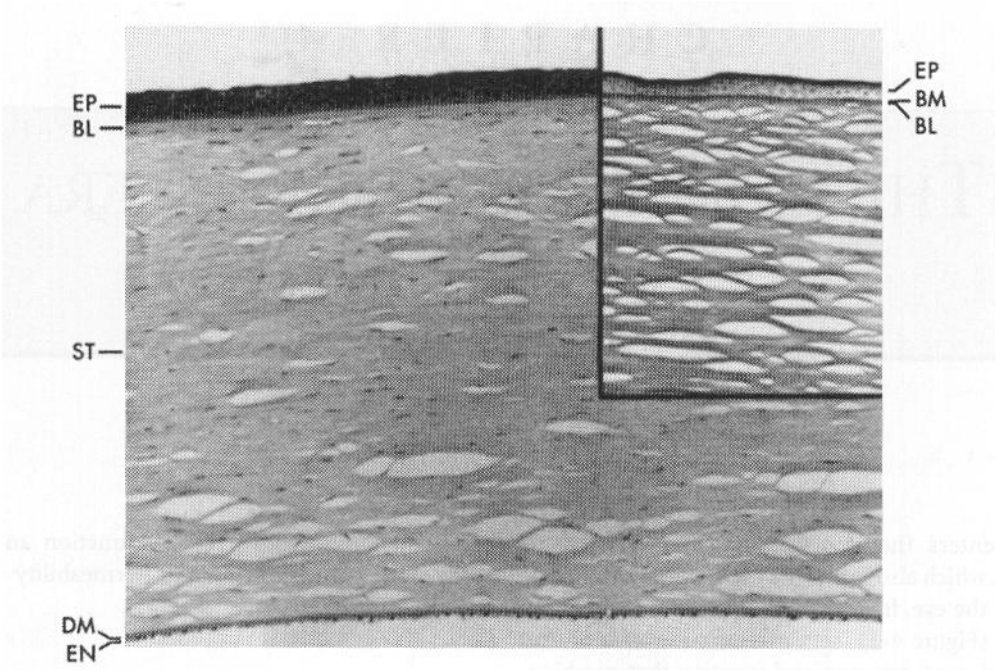

Figure 1.3: Histological section of a normal human cornea stained with Periodic Acid Schiff reveals the outer epithelium (EP), basement membrane (BM), Bowman's layer (BL), stroma (ST), Descemet's membrane (DM), and endothelial monolayer (EN). (9)

aqueous humor: diffusion, ultrafiltration and active secretion (5). Diffusion involves the passive flow of solutes across a cell membrane down a concentration gradient. Ultrafiltration refers to the bulk flow of blood plasma from the ciliary capillary into the ciliary stroma. This process can be aided by the utilization of the hydrostatic driving force (6). Active secretion involves the movement of a solute across a membrane against the concentration gradient, at the cost of cellular ATP.

The first two processes are responsible for the formation of the reservoir of plasma ultrafiltrate in the stroma. By active secretion, the plasma ultrafiltrate is then pumped into the posterior chamber of the aqueous humor. This active secretion process accounts for the formation of 80-90% of the total aqueous humor (18). In a healthy human eye the rate of aqueous humor formation is approximately 2.5 $\mu l \times min^{-1}$ (19).

1.2.4 Vitreous Humor

The vitreous humor is the largest structure in the eye, making up 80-90% of its total volume (9). Physically, the vitreous is located behind the lens and extends all the way back to the retina (Fig 1.4) (20). Water makes up 99% of the vitreous' composition. The rest is made up of solids. The vitreous acts as a gel to surround the large amount of water. The gel portion consists of long, thick, non-branching, collagen fibrils suspended in a network of hyaluronic acid which stabilizes the gel structure and the conformation of the collagen fibrils (21).

The gel structure acts as a barrier against the movement of solutes. Substances may, however, travel in and out of the vitreous via diffusion and bulk flow. Bulk flow influences the movement of high-molecular-weight substances only, and is facilitated by the flow of liquid from the retrozonular space (anterior) to the retina (posterior) (22). Movement across the blood-retinal barrier is restricted to low-molecular-weight substances and occurs via diffusion; similar to that of the blood-brain barrier (23).

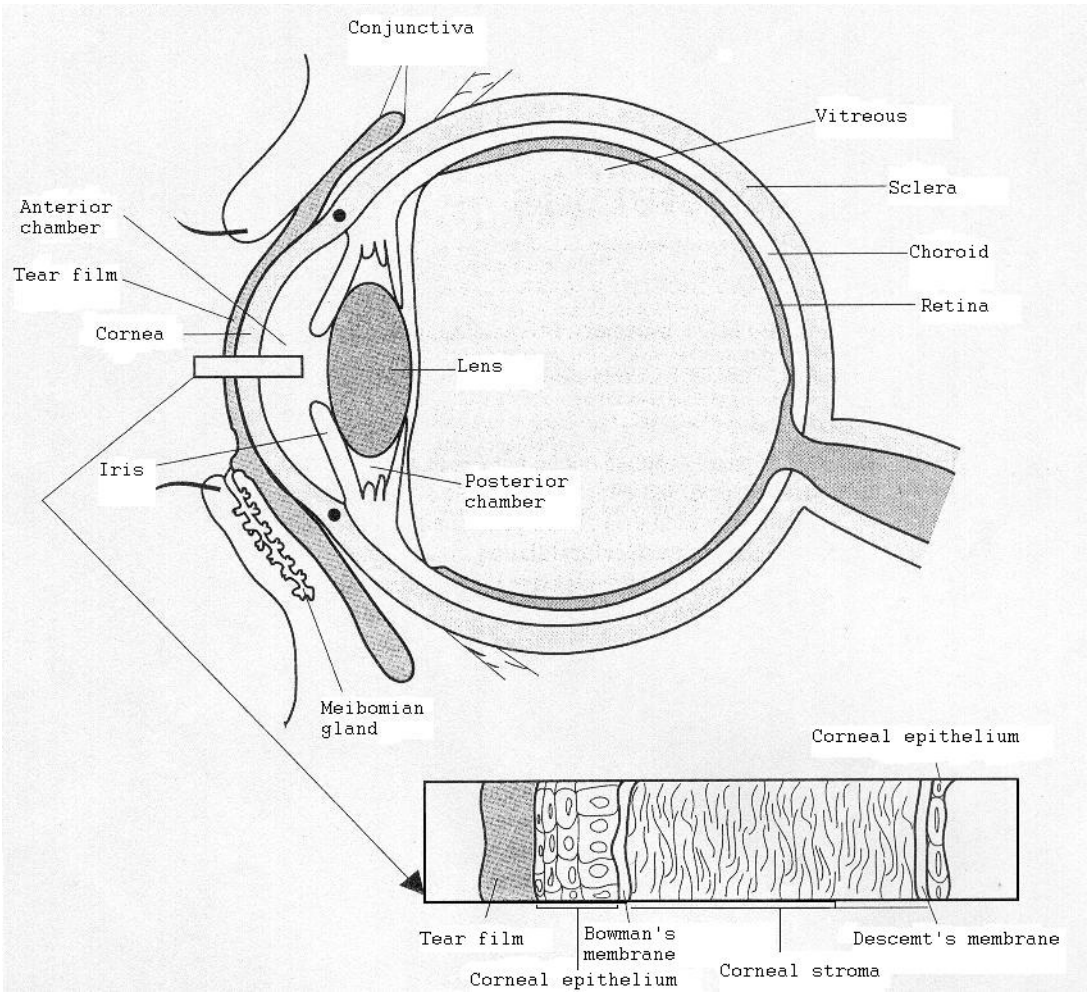

Fig 1.4: A cross-sectional view of the eye. (20)

1.3 Bacterial Ocular Infections

1.3.1 Conjunctivitis (Pink-eye)

The conjunctival resident bacteria, or normal flora, tends to fluctuate with the age and physical state of the host, as well as environmental factors (2). Typically adults harbor a greater number of organisms than do children. Children are colonized with higher numbers of *Streptococcus species* while adults have higher numbers of anaerobes such as *Propionibacterium species* (24). Also, long-term contact lens wearers have higher number of bacterial species than do short-term and non-contact lens wearers (25). While the presence of normal flora acts as a natural defense mechanism by out-competing pathogenic bacteria, normal flora may cause infection in immunocomprimised or debilitated patients.

Realistically any microbial organism can lead to conjunctivitis. However, the most common bacterial pathogens are *S. aureus*, *H. influenzae* and *S. pneumoniae* (2) (Table 1.1). The method of transmission can be any of the following: hand to eye, person to person, contaminated instruments or surfaces and inadvertent sexual contact. Symptoms usually include chemosis, hyperemia and discharge/exudates. The severity of the symptoms depends on the infectious agent present, and the type of immune response elicited (2). For example, when exudate is present, it may be serous, mucoid, purulent or hemorrhagic (26).

Conjunctival membranes or pseudomembranes may be present as a result of infection. Both consist of fibrin and cellular debris. The true membranes actually attach to the conjunctival epithelium. As a result, when removed, the membranes leave a raw and bleeding surface. In contrast, the pseudomembranes are not attached to the conjunctival surface, so when removed there is no trauma (26). As with the type of exudates present, the type of membrane can help establish a differential diagnosis.

Similar to other infections, the symptoms are created mostly by the immune response. This consists primarily of an infiltration of lymphocytes, neutrophils, mast cells and plasma cells which coincides with an increase in the ocular vascular permeability (27).

Type of Infection	Organism	Prevelance (%)	Reference
Conjunctivitis	**Gram - positive**	75.0	Everett *et al*. 1995
	Coagulase Negative *Staphylococcus*	39.0	
	S. aureus	25.0	
	Gram - negative	25.0	
	H. influenzae	9.0	
	Enteric Gram - negatives	5.4	
Pediatric Conjunctivitis	*H. influenzae*	20 to 75	Block *et a*l. 2000
	S. pneumoniae	12 to 20	
Keratitis	**Gram - positive**	48.0	Alexandrakis *et al.* 2000
	S. aureus	19.4	
	Streptococus species	6.7	
	Diptheroids	2.3	
	S. epidermidis	1.3	
	Gram - negative	50.0	
	P. aeruginosa	25.7	
	Serratia marcescens	7.6	
	Proteus species	3.7	
	Haemophilus species	2.5	
Endophthalmitis	**Gram - positive**	37.6	Anand *et al*. 2000
	S. epidermidis	12.9	
	S. aureus	7.6	
	P. acnes	5.9	
	Streptococcus species	4.1	
	Gram - negative	41.7	
	P. aeruginosa	17.1	
	Other Gram - nagative bacilli	10.6	
	Other non-fementing bacilli	8.8	

Table 1.1: Prevalence rates of bacteria most commonly associated with conjunctivitis, keratitis and endophthalmitis.

While mild conjunctivitis is often self-limiting, treatment is sometimes given to reduce patient anxiety, prevent spread to the other eye and shorten the duration of the disease (28). Treatment consists of a topical broad spectrum antibiotic such as an aminoglycoside or a fluoroquinolone given four times daily for five to seven days. Moderate conjunctivitis requires more frequent doses such as six to eight times daily for seven to ten days. Treatment for severe acute conjunctivitis will last for seven to fourteen days and may include concurrent systemic treatment if preseptal cellulitis or otitis media are involved. This is especially important for children if *H. influenzae* is the causative organism (2).

1.3.2 Keratitis

Because of its high incidence and potential complications, bacterial keratitis is one of the most visually threatening ocular infectious diseases. The avascular cornea is particularly susceptible to bacterial infection, and many patients have a poor clinical outcome it aggressive therapy is not initiated promptly (29). Organisms such as *S. aureus* and *P. aeruginosa* have been known to create corneal perforations in less than 24 hours (30).

Normal flora for the cornea consists primarily of *S. epidermidis* and diptheroids, but also *S. aureus*, *S. pneumoniae*, *P. aeruginosa* and *Neisseria meningitidis* (31) (Table 1.1). The primary pathogens are *S. aureus*, *S. pneumoniae* and *P. aeruginosa*.

Corneal infections result mostly from a failure of one of the host defense mechanisms. Most bacteria cannot penetrate an intact corneal epithelium, with the exception of *Neisseria gonorrheae* and *H. influenzae*, because it is protected by eyelids and tear film (15). Consequently, it takes a condition such as dry eye, or trauma to the cornea to facilitate the development of a bacterial infection. The advent of contact lenses has led to a significant (15-20-fold) increase in the incidence of ulcerative conjunctivitis (32). This is because the cornea epithelial cells receive micro-abrasions every time a contact lens is inserted or removed. This creates additional opportunities for pathogens, especially *P. aeruginosa*, to invade (33). Additional risk factors include: poor personal hygiene, diabetes mellitus, ocular steroid use, recent ocular surgery and use of contaminated ophthalmic solutions (34).

Symptoms of keratitis often include a rapid onset of pain, conjunctival injection, decreased vision, photophobia and white cell infiltrate (20). Other conditions such as uveitis, edema and opacification may also appear. Corneal infections result in the release of prostaglandins and other chemical mediators. A combination of prostaglandin-mediated miosis, breakdown of the blood-aqueous barrier and vasodilation contribute to the development of anterior uveitis (35). Edema may also be present. If the damage remains at the epithelial level, the edema may be quite localized. If the damage extends down to the endothelial layer, however, the edema may be quite diffuse. Lastly, inflammatory cells may enter the cornea via limbal vasculature or tear film in response to antigens, toxins or other irritants. This presents as cloudy corneal infiltrates (36).

Treatment of infectious keratitis must occur swiftly upon diagnosis and the patient's progress must be closely monitored. While specimens are not usually collected in cases of conjunctivitis, it is important to do so for keratitis. While waiting for the lab results, treatment via combination therapy or a monotherapeutic agent needs to occur. Combination therapy refers to the use of two antimicrobial agents to get better Gram-positive and Gram-negative coverage. This usually involves combining an aminoglycoside, such as tobramycin, and a cephalosporin like cefazolin. Monotherapy refers to the use of a single antimicrobial agent which has sufficient spectrum of activity to be used alone. An example of a monotherapeutic agent would be any of the ophthalmic fluoroquinolones.

For the first 24 hours of treatment, installation of the antibiotic needs to occur hourly. If the response is favorable then the frequency can be reduced to 2-hourly. If progress is not being made (ex. ulceration progressing), the physician can refer to the lab results to determine what antibiotic would most likely be effective based on *in vitro* susceptibility results (15).

1.3.3 Endophthalmitis

Bacterial endophthalmitis can develop a few different ways. It is most often a postoperative infection but it can also arise from a corneal ulcer which perforates the cornea, or a penetrating trauma. The source of infection is usually the patient's own eyelid, conjunctival or lacrimal flora. It may sometimes come from contaminated surgical solutions, instruments or environmental flora (15). Common pathogens include *S. epidermidis*, *S. aureus*, *Pseudomonas* species and *Proteus* species (Table 1.1).

Clinical features of mild endophthalmitis include slight pain, preservation of some red reflex (the natural color reflected off of a healthy retina), and possibly a small hypopyon (presence of leucocytes). Moderate endophthalmitis results in fibrinous exudates in the vitreous, small hypopyon, vitritis and an absence of red reflex. Severe endophthalmitis presents with pain, significant visual loss, lid edema, chemosis, conjunctival injection, purulent discharge, corneal infiltrates and large hypopyon (15).

Prior to initiating treatment, vitreous and aqueous specimens should be collected and sent to the lab. This should consist of a 100 µl aqueous aspirate and a 100-300 µl aspirate from the vitreous. Treatments vary depending on the severity of the infection. One option is to use intravitreal injections. Like other types of ocular infections, this is done using a fluoroquinolone or a combination of agents. This is done once daily for five to seven days. One problem with this type of therapy is that some drugs show retinotoxicity, which can result in additional sight loss. Aminoglycosides are particularly noted for this (15). Despite this, intravitreal injections are the fastest way to administer drugs to the vitreous. Another method of treatment is topical therapy. This has limited effectiveness because unless there is a damaged corneal epithelium, as with infectious keratitis, the topical drugs either take a long time to reach bactericidal concentrations in the vitreous or they do not reach bactericidal concentrations at all. The last option is systemic treatment. This usually is not recommended due to poor ocular penetration. Ocular drug concentrations only reach approximately 10-20% of serum concentrations (37). Despite aggressive therapy 55% of eyes achieve a final visual acuity of 6/60 or less (15). In severe cases where visual acuity is reduced to light perception, a vitrectomy will be performed.

1.4 Antimicrobial Therapy for Bacterial Ocular Infections

1.4.1 Topical/Systemic

Typically for most ocular infections, with the exception of endophthalmitis, the two treatment options are either topical or systemic antimicrobial therapy. As mentioned above, systemic treatment alone is not recommended because of the poor ocular penetration. The poor penetration has two consequences. First, bactericidal concentrations at the site of infection are not achieved which may result in therapeutic failure. Second, exposing bacteria to non-bactericidal drug concentrations may promote the selection of antimicrobial resistant bacteria.

Topical therapy is very successful when used to treat superficial ocular infections like conjunctivitis and keratitis. Drug concentrations greatly exceeding the minimum inhibitory concentration (MIC) for most organisms are achievable. This is because the drug can be directly delivered to the site of infection instead of having to pass through the blood-ocular-barrier and cornea (20).

1.4.2 Tear Film Dilution

One complicating factor with respect to topical application is the tear dilution. Each drop of antibiotic has a volume of 40-50 µl. Because the conjunctival cul-de-sac only has a capacity for 20-30 µl, half of the drop volume is spilled from the eye upon instillation. Between two to five minutes after the drop has been administered, the tear film will reduce to a normal volume of 7 to 9 µl. Reflex tearing and blinking induced by instillation increases spillage onto the skin. Some drug will also be lost via drainage through the nasolacrimal duct to the nose where it will be ingested. The average tear production of 1 µl/minute will continue to dilute the drug resulting in a half life of 2-20 minutes (38). Consequently, multiple doses are needed to keep the drug concentrations above the MIC.

Instilling drops at intervals less than the five minutes it takes for the tear film to reach its normal volume results in a disproportionate loss of drug from the eye. In circumstances where two drugs are being used for treatment, and are administered separately, the first agent will be displaced from the eye with rapid addition of the second agent (39).

1.4.3 Barriers

Topical application for treatment of intraocular infections like endophthalmitis is more complicated because, in addition to dealing with the tear dilution, the drug has to diffuse through the cornea. The three main layers which present the problem are the epithelium, stroma and endothelium. Factors affecting the rate of diffusion are the drugs' lipid and water solubility, molecular size and ionization. Drugs that are highly lipophilic penetrate the epithelium easily, but are slowed down by the hydrophobic stroma (20).

Many antibacterial agents like chloramphenicol, and the fluoroquinolones, when applied frequently, penetrate the cornea sufficiently to achieve therapeutic concentrations for most organisms. Unfortunately, accumulation of high concentrations of drug is prevented by the flow of aqueous humor from the ciliary process, where it is secreted, through the posterior and anterior chambers, and out of the eye via Schlemm's canal (20). Consequently treatment of endophthalmitis may consist of topical and systemic antibiotic, or topical and intravitreal application.

1.4.4 Resistance

The development of bacterial resistance to antimicrobial agents has become a global concern. Infections caused by antimicrobial resistant bacteria are associated with higher rates of hospitalization, greater length of hospital stay, and higher rates of illness and death (40). Antimicrobial resistance develops when bacteria are exposed to an antimicrobial agent, and selective pressure favors the growth of resistant organisms (41).

While increasing resistance rates have been observed in all of the organisms most commonly associated with ocular infections, the rate of antimicrobial resistant Gram-positive pathogens has increased the most. Currently 25 – 30% of strains of *S. pneumoniae* in the United States have reduced susceptibilities to penicillin. Of those 25 – 30%, approximately 40% have intermediate resistance and 60% have full resistance (42). Cross-resistance to third-generation cephalosporins, macrolides, co-trimoxazole, and tetracycline increases progressively in penicillin-intermediate and penicillin-resistant strains (43). It was shown in another study that ocular isolates collected from 1990 – 1998 revealed the *S. pneumoniae* susceptibility to fluoroquinolones

(ciprofloxacin and ofloxacin) and aminoglycosides (tobramycin and gentamicin) was as low as 70% and 17% respectively (44).

Similar trends have been observed with *S. aureus*. For example over a period of 12 years, the resistance rates for ciprofloxacin against methicillin-sensitive *S. aureus* (MSSA) isolated from conjunctival and corneal specimens rose from 8% to 20.7% (45). Another published study reported *S. aureus* susceptibility rates of 78 – 94% for gentamicin, 83 – 89% for tobramycin, 92 – 98% for ciprofloxacin and ofloxacin, and 94 – 96% for cephalothin on corneal and conjunctival isolates collected from 1997 to 2000 (46).

Gram-negative ocular pathogens like *P. aeruginosa* and *H. influenzae* have not had rates of resistance increase to the same degree as Gram-positive organisms. Against common ocular antimicrobials such as tobramycin and gentamicin, both of which are aminoglycosides, *P. aeruginosa* susceptibilities as low as 92% and as high as 100% have been reported(46). With fluoroquinolones, ciprofloxacin and ofloxacin, susceptibility for *P. aeruginosa* ranges from 91% (46) to 100% (47). *H. influenzae*, similarly, has maintained very high susceptibility rates to the fluoroquinolones (48).

Because fluoroquinolones are an important class of antimicrobial agents, and in response to increasing resistance primarily in Gram-positive organisms, two new fluoroquinolones have been licensed since 2003 for ophthalmic use. Moxifloxacin was introduced in April of 2003 in the United States with the trade name Vigamox, and is available as a 0.5% ophthalmic solution. Gatifloxacin was introduced in March of 2003 in the United States with the trade name Zymar, and is available as a 0.3% ophthalmic solution.

1.5 Fluoroquinolones

1.5.1 General Overview

Naldixic acid, the first quinolone, was introduced in 1962. It had good *in vitro* Gram-negative activity and was used primarily to treat uncomplicated urinary tract infections. Since then, structural modifications to the core molecule have been made in attempts to increase the spectrum of activity of the subsequent fluoroquinolone compounds. This has resulted in the development of four generations of fluoroquinolones which have become important in the treatment of bacterial infections.

Changes made to the naphthyridone nucleus of naldixic acid, such as a piperazine substitution at the R_7-position and fluorination at the R_6-position, (Figure 1.5) lead to the development of second generation drugs like ciprofloxacin and ofloxacin in the late 1980s and early 1990s (49). They were characterized as having broader Gram-negative coverage, the ability to achieve high systemic concentrations and had some atypical pathogen coverage. Ciprofloxacin, especially, is very active *in vitro* against clinical isolates of *P. aeruginosa*. Second generation agents are indicated for treatment of upper and lower respiratory tract infections, uncomplicated urinary tract infections, sexually transmitted diseases (STD's), nosocomial infections, gastroenteritis and prostatitis. Use for treatment of community acquired pneumonia is not recommended due to the risk of pneumococcal bacteremia and meningitis (50).

Classification of third and fourth generation agents is somewhat debated. With regards to systemic treatment, levofloxacin, gatifloxacin and moxifloxacin are considered third generation agents, while trovafloxacin is the sole fourth generation agent (50). In ophthalmic circles, however, the designations are slightly different. Levofloxacin is a third generation agent while both moxifloxacin and gatifloxacin are labeled as fourth generation agents (51).

Structurally, levofloxacin is the L-isomer of ofloxacin. While its Gram-negative activity is comparable to second generation agents like ciprofloxacin and ofloxacin, it is slightly more active against certain Gram-positives and atypical pathogens (52). Fourth generation agents were engineered to deal with increasing fluoroquinolone resistance. As a result a methoxy side-chain was added to both moxifloxacin and gatifloxacin at the R_8 position. Moxifloxacin also has a bulky bicyclic ring attached at the R_7 position while gatifloxacin has a methyl group on its piperazinyl ring (51). These changes resulted in increased *in vitro* activity (i.e. lower MICs) against *Streptococcus* and *Staphylococcus* species. At the same time, these compounds have been shown to be able to kill organisms that had shown resistance to previous fluoroquinolone compounds.

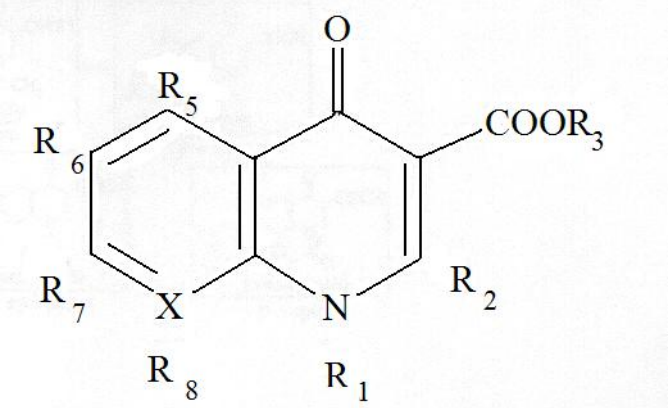

Figure 1.5: Basic quinolone skeleton (4-oxo-1, 4 dihydroquinolone) (51)

In the 1990's, second and third generation fluoroquinolones were licensed for ophthalmic use (52). Their broad spectrum activity made them good selections for first-line topical therapy for infections like conjunctivitis and keratitis. Ciprofloxacin and ofloxacin were considered comparable to popular ocular agents like chloramphenicol 0.5%, tobramycin 0.3% and gentamicin 0.3% for the treatment of conjunctivitis, except that the fluoroquinolones were able to reduce the signs and symptoms of infection more rapidly (53) (54).

With regards to aqueous and vitreous penetration, it was shown in one study that following a dosing regimen of one drop/15 min. x 5, then one drop/30 min. x 3 for both ciprofloxacin and ofloxacin resulted in aqueous concentrations of 1.13 μg/ml and 2.06 μg/ml respectively. Vitreous concentrations reached 0.23 μg/ml for ciprofloxacin and 0.46 μg/ml for ofloxacin (55). It should be noted that these results were conducted on patients with uncompromised corneas. In cases with corneal ulceration, aqueous and vitreous drug concentrations will be even higher. Drug concentrations in the tear film are much higher. LC Green *et al*. (56) showed that, following the installation of two drops of ciprofloxacin, the drug concentration reached 2203 μg/ml at the five minute interval and dropped to 17.9 μg/ml after 30 minutes .

1.5.2 Mechanism of Action

Fluoroquinolones act on two bacterial enzymes, DNA gyrase (topoisomerase II) and topoisomerase IV, which are both involved in bacterial DNA sythesis (51). DNA gyrase is composed of two GyrA, and two GyrB monomeric subunits encoded by *gyrA* and *gyrB* respectively. It is responsible for creating negative supercoils by relieving positive supercoils ahead of the replication fork. This catalyzes the separation of daughter chromosomes; a process essential for replication (57). Topoisomerase IV is composed of four homologous monomeric subunits, two ParC subunits and two ParE subunits encoded by *parC* and *parE* respectively (58). This enzyme is required to separate linked daughter DNA molecules after replication is complete (59).

The enzyme specificity of second and third generation agents depends on the type of bacteria being targeted. For example, against Gram-positive bacteria, topoisomerase IV is the primary target whereas DNA gyrase is the primary target for Gram-negative bacteria (60). Fourth generation agents have been shown to exhibit dual

activity. They act on both DNA gyrase and topoisomerase IV. This may be important because these agents are able to kill bacteria possessing single mutations of either DNA gyrase or topoisomerase IV (61).

1.5.3 Mechanism of Resistance

The fluoroquinolones are subject to two main mechanisms of bacterial resistance: alterations in the target enzyme binding sites, and efflux pumps. Alterations, via point mutations, in DNA gyrase most commonly occur in fluoroquinolone-resistant Gram-negative bacteria (62). Mutations can affect either the GyrA or GyrB subunits but GyrA is more common (51). Mutations tend to occur within a certain region of the gene known as the quinolone resistance-determining region (QRDR). The QRDR is the region which encodes the portion of the GyrA subunit which binds to DNA during enzyme activity (63). Mutations in topoisomerase IV affect either the ParC or ParE subunits, however, ParC is more common (64). Like the *gyrA* and *gryB* genes, the topoisomerase point mutations in *parC* and *parE* usually occur in the QRDR. These mutations are most common in fluoroquinolone-resistant Gram-positive bacteria (51). Due to the single target action of second and third generation fluoroquinolones, single mutations significantly affect the potency of these drugs. Fourth generation fluoroquinolones, however, are not as effected due to their dual target activity (65).

Efflux-mediated resistance is present in bacteria that possess special membrane complexes which are capable of actively pumping fluoroquinolones out of the cell. This allows the bacteria to survive in the presence of the drug by reducing the intracellular concentration to sublethal levels. The pump's action is dependent on its ability to bind to the fluoroquinolone (66). This was considered an important factor when developing fourth generation fluoroquinolones. For example, due to the bulky side-chain added to moxifloxacin, it is less effected by efflux mechanisms (67).

1.5.4 Pharmacokinetics and Pharmacodynamics

Because antimicrobial resistance has become a huge concern, healthcare professionals need to ensure that the right drugs are being used for the appropriate cases and formulary committees need to ensure that they are recommending effective dosing

regimens. To help achieve this, certain drug characteristics, like the pharmacokinetics (PK) and pharmacodynamics (PD) can be analyzed .

Fluoroquinolones are considered to exhibit concentration-dependent killing, which means that as the concentration of drug increases, so does the killing effect (68). Other PK properties including the concentrations of drug in the serum over time, referred to as the area under the curve (AUC), and the peak serum concentration of the drug (C_{max}), can be measured. When these values are considered in combination with *in vitro* activity, predicting the microbiological and clinical outcome may be possible. Of particular interest are the ratios of either C_{max} to MIC or AUC to MIC resulting in the area under the inhibitory curve (AUIC) (69) (Fig 1.6). Studies have identified the minimum AUIC values necessary to achieve the desired antimicrobial activity and clinical efficacy. Higher AUIC values, such as 125, have been shown to reduce the emergence of resistance while even higher values have been shown to quicken bacterial eradication (70). Another study done by Thomas *et al*. suggested that AUC/MIC should exceed 100 for both Gram-negative and Gram-positive species to prevent the development of resistance (71).

1.5.5 Side Effects

With systemic use of fluoroquinolones there is a low incidence of side effects which include: nausea, headache, dizziness, rash, bitter taste, an elevation of liver enzymes and eosinophilia (2). A study was done by HM Leibowitz (72) which investigated the side effects associated with topical ciprofloxacin therapy for 1500 cases of bacterial keratitis. The following incidences of side effects were noted: 9.7% had an ocular burning sensation, 5% had bitter taste, 3.6% developed a white precipitate, 2% had a foreign body sensation, 1% experienced itching and <1% developed conjunctival hyperemia, chemosis and photophobia.

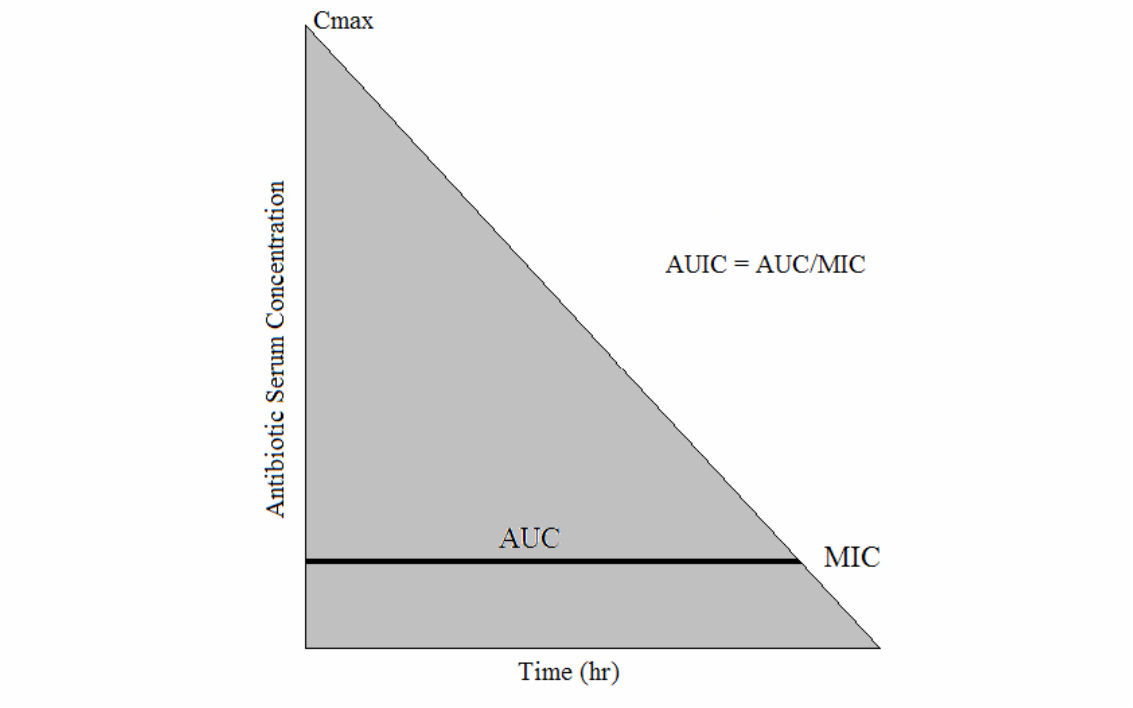

Figure 1.6: The AUC is a universally derived formula used to predict the clinical success and development of resistance based on the relationship between clinical PK and the MIC of the particular pathogen. The area created between the serum concentrations of the drug over time and MIC for a given drug and organism defines the AUC (73).

1.6 Determining Antimicrobial Susceptibility

1.6.1 Minimum Inhibitory Concentration (MIC)

In order to ensure that antibiotics are being prescribed for organisms that are susceptible, routine susceptibility testing is conducted. This is done by collecting a specimen and sending it to the clinical laboratory so that a pathogen may be recovered, identified and the MIC determined. The MIC is defined as the lowest concentration of drug needed to inhibit bacterial growth when 10^5 colony forming units per milliliter (cfu/ml) are exposed to varying drug concentrations. Based on the results from the MIC test, the isolate is classified as susceptible, intermediate or resistant based on guidelines set up by the Clinical Laboratory Standards Institute (CLSI formerly NCCLS). The physician, upon receiving the results, can then decide to continue the current treatment or switch to a drug that is more suitable for treating the infecting pathogen(s).

Despite being a globally standardized and accepted test of susceptibility, there are some problems with MIC testing which may, in fact, be aiding the development of resistance. The main problem has to do with the size of the inoculum of approximately 10^5 cfu/ml, as in various human infections bacterial loads may be $\geq 10^9$ cfu (74). This becomes important when taking into account the mutation frequency of bacteria. It has been reported that there is approximately one mutation for every $10^7 – 10^9$ cells (75). With this in mind, various human infections may contain at least one resistant bacterium based on spontaneous mutations. However, with an antibiotic dosing strategy based on MIC results, only the susceptible bacteria are taken into consideration. With drug concentrations that kill susceptible organisms but not resistant bacteria, drug dosing may be actively selecting or enriching the resistant sub-populations (76).

1.6.2 Mutant Prevention Concentration (MPC)

The MPC is a new type of susceptibility test that represents an attempt to compensate for the shortcomings of MIC testing (65). The MPC concept is particularly relevant for the fluoroquinolones, as mutations occur in a step-wise manner (77). First-step mutations occur at a frequency of 10^{-7} - 10^{-9}, and a second-step mutation occurs with a frequency of 10^{-14} or lower (75). Using an inoculum of 10^{10} cfu/ml (or $\geq 10^9$ cfu/ml for *S. pneumoniae*) which is dispensed onto agar plates with drug incorporated into the media, the MPC is able to more accurately approximate the concentration of

drug needed to eradicate a bacterial population containing both susceptible, and first-step resistant bacteria (Fig 1.7). Consequently, the definition of MPC is the "concentration of antimicrobial agent that would require an organism to possess two concurrent mutations to grow in the presence of that drug" (65). Theoretically, if dosing strategies were to be based on the MPC concept, it would be easier to maintain the efficacy of the fluoroquinolones because resistant bacterial populations would be eliminated before they could develop into second-step mutants; organisms containing two concurrent mutations are resistant to all available fluoroquinolones.

1.7 *In vitro* Growth Dynamics

1.7.1 Kill Curves

While the MIC and MPC experiments determine what drug concentrations are required to inhibit growth of susceptible bacteria and bacteria with first-step resistance mutations respectively, they do not provide any information on bacterial killing. As a result, another experimental approach is required to determine how long after exposure to an antimicrobial agent it takes for a significant bacterial population reduction to occur and how long this effect is maintained. Kill curves are one approach which allows for the investigation of bacterial killing. Because different locations on the body facilitate bacterial growth of varying loads (ex. ocular loads can reach $10^6 - 10^7$ cfu (78), respiratory loads can reach 10^{10} cfu (79), kill curves are conducted with an inoculum size ranging from 10^6 to 10^9 cfu/ml. Because different antimicrobial agents can be tested against different bacteria, comparisons of each drug's ability to reduce different pathogens can be established.

1.8 Summary

Because, as humans, our lives depend so heavily on the use of our visual senses, any degree of damage to the visual system can have a huge impact on our quality of life. While bacterial eye infections do not occur with great frequency, they need to be taken very seriously. Since infections can occur in every structure in the eye from the conjunctiva in the anterior portion to the vitreous humor in the posterior portion, we need to ensure we are equipped with antibiotics capable of reaching and then killing the infecting pathogen.

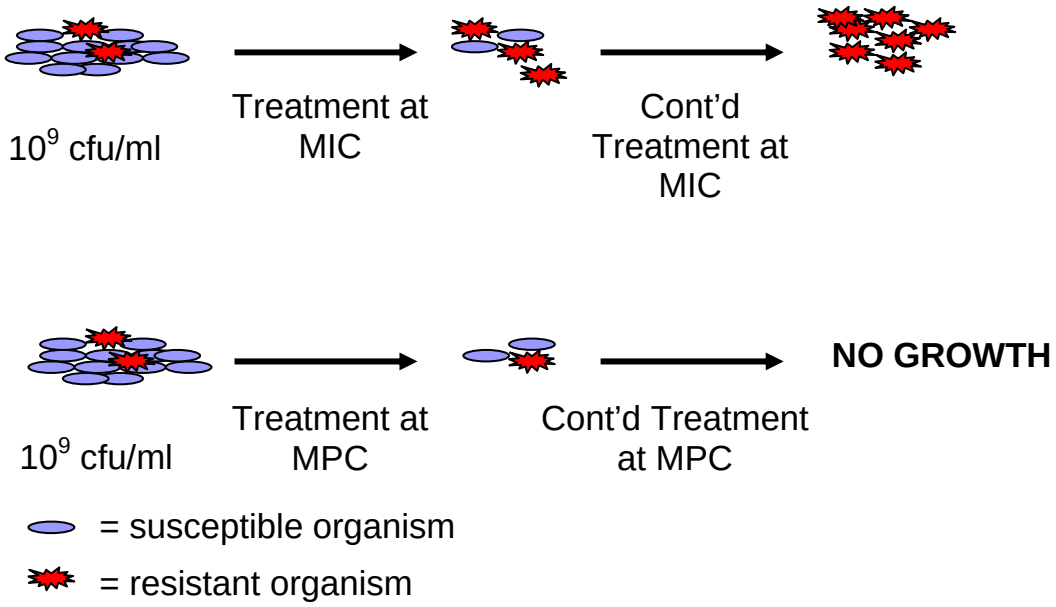

Figure 1.7: First-step resistance mutants are present in bacterial cultures of $10^7 - 10^9$ cfu/ml. Treatment at the MIC reduces only susceptible cells resulting in an enrichment of resistant organisms. Treatment at the MPC should reduce both the susceptible and resistant populations, thereby avoiding a selective enrichment of resistant sub-populations as seen with treatment using lower drug concentrations.

The fluoroquinolones are a class of antimicrobial agents that have become excellent choices for use in treating ocular infections. However, with extensive systemic use, improper dosing and a lack of patient compliance, rates of bacterial resistance have increased. In response to the increasing resistance, particularly amongst Gram-positive pathogens, researchers have developed two new fluoroquinolones, gatifloxacin and moxifloxacin. However, in order to convince physicians that these two new drugs will be a successful substitute for the existing fluoroquinolones, it needs to be shown that moxifloxacin and gatifloxacin have better spectra of activity while maintaining the ocular penetration of ofloxacin and ciprofloxacin, and the ability to attain a bactericidal effect in a short period of time.

1.9 Objectives

In order to address the above questions with regards to moxifloxacin and gatifloxacin, three objectives were established. The first objective was to determine the MICs for four typical ocular pathogens (*S. pneumoniae*, *S. aureus*, *H. influenzae* and *P. aeruginosa*) and two atypical pathogens (*Mycobacterium fortuitum and Mycobacterium chelonae*) against five fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin and gatifloxacin. The second objective was to determine the MPCs for the same organisms against the same drugs. The last objective was to set up kill curves for five fluoroquinolones against isolates of a representative Gram-positive organism (*S. pneumoniae*) and of a representative Gram-negative organism (*H. influenzae*). The kill curves were investigated at both the MIC and MPC drug concentrations and against a range of inocula from $10^6 – 10^9$ cfu/ml.

2.0 Materials and Methods

2.1 Standard Laboratory Methods

2.1.1 Isolate Collection and Identification

The isolates used in this study were all ocular isolates with the exception of the *Mycobacterium species* isolates. The *Streptococcus*, *Staphylococcus*, *Pseudomonas* and *Haemophilus* isolates were collected through the Clinical Microbiology Laboratory at Royal University Hospital as part of a provincial Health Services Utilization and Research Commission (HSURC) study in 2000. Some additional *Staphylococcus*

ocular isolates were kindly provided by Dr. T. O'Brian, Wilmas Eye Institute John Hopkins Medical Center (Baltimore, Maryland). All isolates were collected and identified using reference laboratory methods.

2.1.2 Isolate storage

All isolates used were sub-cultured onto Tryptic Soy Agar (TSA) plates containing 5% sheep blood (*H. influenzae* onto Chocolate agar plates and *Mycobacterium* species onto Middlebrook 7H11 agar plates), incubated at optimum temperature (35 - 37 °C) and atmospheric conditions for *S. aureus* and *P. aeruginosa* (5% CO_2 for *Streptococcus*, *Haemophilus* and *Mycobacteria*) for 18 - 24 hrs. The cultures were then transferred to a 1.2 ml Corning cryovial containing 0.5 ml of skim milk via a sterile wooden stick applicator. The cryovials were then stored at -70 °C.

2.2 Susceptibility Testing

2.2.1 Minimum Inhibitory Concentration

The susceptibility testing for this project was done using the broth microdilution technique as recommended by the CLSI handbook (80)). Cultures were inoculated onto fresh agar plates and incubated at the appropriate atmospheric conditions and temperature for 18 - 24 hrs. One hundred µl of the appropriate broth was added to rows 2-12 of a ninety-six well flat bottom microtitre plate (Mueller Hinton Broth (MHB) for *Staphylococcus* and *Mycobacteria*, Todd Hewitt Broth (THB) for *Streptococcus*, Pluronic Inoculum water for *Haemophilus*). Two hundred µl of the antimicrobial agent was added to row 1 and then serially diluted through row 10. No drug was added to row 11 which served as a growth control. Each isolate was standardized to a 0.5 McFarland standard using a colorimeter ($\approx 1.0 \times 10^8$ cfu/ml). The bacterial suspension was diluted 1/100 using the appropriate broth (as above except Brain Heart Infusion (BHI) plus 5% fildes for *H. influenzae*) to achieve a final concentration of $\approx 1.0 \times 10^6$ cfu/ml. One hundred µl of bacterial cells was added to each well on the panel resulting in a final volume of 200 µl. The purity of each isolate was checked by taking a sample of each isolate added to the microtitre panel and inoculating a fresh agar plate. Both the purity plates and the microtitre panels were then incubated as described. The microtitre panels were removed from the incubator after 16-20 hrs and read. The results were considered valid if the American Type Culture Control (ATCC) strains were within the

susceptibility limits established by CLSI guidelines. The ATCC strains used were *S. pneumoniae* 49619, *S. aureus* 29213, *P. aeruginosa* 27853 and *H. influenzae* 49247.

2.3 Mutant Prevention Concentration

Protocols for *S. pneumoniae*, *S. aureus* and *P. aeruginosa* were previously established (81, 82). Protocols for *H. influenzae*, *M. chelonae* and *M. fortuitum* were developed as part of this thesis work. Each organism had a corresponding ATCC control strain tested with initial experiments. For example, the *S. pneumoniae* ATCC strain 49619 was tested with the *S. pneumoniae* isolates.

2.3.1 Inoculum Preparation

In order for this test to be run successfully, each isolate must be grown to a concentration $\geq 1.0 \times 10^9$ cfu/ml. This was done slightly differently for each organism due to the differing growth characteristics. Development of MPC protocols for testing *H. influenzae* and *Mycobacteria* involved several different steps. First, literature searches were done to determine what types of media, liquid and solid, would work best for growing these organisms. For *H. influenzae* this was HTM broth and agar, for *Mycobacteria* it was MHB with 0.02% Tween 80 for broth and MH agar supplemented with OADC enrichment and 5% glycerol. Tween and glycerol were used to keep the *Mycobacteria* from clumping together. Next, and most importantly, I needed to determine how to consistently get growth at $\geq 10^9$ cfu/ml. Aliquots of broth at 100, 200, 300, 400 and 500 ml had two plates with confluent bacterial growth transferred into them and incubated for 24 hours with 5 % CO_2 at 35-37°C. The same set of broth aliquots were set up using three plates to inoculate from, and another set of broth for four, five and six plates. After incubation viable counts were done in triplicate from each bottle of broth. If bacterial counts were too low, then the samples were centrifuged and the pellets were re-suspended in 5 ml of broth. Viable counts were then done on the concentrated samples. For *H. influenzae* it was found that three plates of bacterial growth added to 100 ml HTM (incubated for 24 hr), then centrifuged for 20 min. and re-suspended in 5 ml of broth gave the desired inoculum size. For *Mycobacteria* it was found that 7.5 plates of bacterial growth added to 100 ml of broth (incubated 72 hr) yielded the correct sized inoculum.

For *S. pneumoniae*, 7.5 blood agar plates were inoculated with organism using a sterile swab to create heavy growth and then incubated for 24 hr as described. The complete contents of the plates were then transferred to 500 ml of THB using sterile swabs, and incubated for another 24 hr as described. A spectrophotometer was used to determine the approximate bacterial population of each sample. A bacterial suspension with an optical density (O.D) ≈ 0.3 at a wavelength of 600nm was required. This reading is equivalent to 1.0×10^9 cfu/ml (Blondeau unpublished observations). The 500 ml samples were then centrifuged at 8000 rpm for 20 minutes at 4°C. The supernatant was discarded and the pellets were re-suspended in 3 ml of fresh THB. Two hundred µl from the 3 ml bacterial suspension was added to each of the drug dilution plates (preparation described in section 2.3.2) and then incubated for 48 hr as described.

For *S. aureus* and *P. aeruginosa*, 2.5 TSA plates were inoculated and incubated for 24 hr as described. The organism was then transferred into 100 ml of fresh MHB and incubated for another 24 hr as described. To achieve the necessary concentration, the bacterial suspension needed to achieve an O.D. reading which was ≈ 1.0 (equivalent to 1.0×10^9 cfu/ml) using a wavelength of 600 nm. One hundred µl from the bacterial suspension was added to each of the drug dilution plates and then incubated for 48 hr as described. The procedure for *H. influenzae* was the same except that chocolate agar plates were used instead of TSA, and *Haemophilus* Test Media (HTM) was used instead of MHB. One difference between both *H. influenzae*, *P. aeruginosa* and *S. aureus*, was that the *H. influenzae* and *P. aeruginosa* 100 ml suspensions needed to be centrifuged for 20 minutes at 8000 rpm at 4°C. The pellets were re-suspended in 5 ml of MHB for *P. aeruginosa* and 5 ml HTM for *H. influenzae*.

M. chelonae and *M. fortuitum* strains were inoculated onto 7.5 blood agar plates and incubated for 72 hr at 35 – 37°C in 5 % CO_2. The cultures were then transferred to 100 ml of MHB supplemented with 0.02% Tween 80 and incubated for 72 hr as described. The bacterial suspension needed to have an O.D. reading ≈ 1.5 at 600 nm (equivalent to 1.0×10^9 cfu/ml). One hundred µl of the suspension was added to each of the drug dilution plates and then incubated for 48 hr as described. MPC values were recorded at 24 and 48 hours.

2.3.2 Agar Drug Dilution Plates

The drug dilution plates were made using the MIC as the lowest drug dilution. The MIC was then doubled six times to get a total of 7 drug concentrations. The amount of drug needed for each plate dilution was calculated with the following formula:

$$C_1V_1 = C_2V_2 \quad (2.1)$$

C_1 refers to the stock concentration of the drug, C_2 is the desired concentration, V_1 is the volume of drug needed to attain the desired drug concentration and V_2 is the volume of the agar to be poured into plates. The plates were made from powdered agar mixed with the necessary ingredients, autoclaved at 121 °C for 15 minutes and then cooled to 55 °C in a water bath. Drug was added to the molten agar, stirred and then poured into sterile petri plates. The volume poured into each plate was approximately 20 ml. The agar for *S. pneumoniae* consisted of TSA plus 5% sheep red blood cells. TSA was used for both *P. aeruginosa* and *S. aureus*. HTM agar was used for *H. influenzae*, and MH agar supplemented with OADC enrichment and 5% glycerol was used for the *Mycobacterium*.

2.3.3 Reading Results

Once the drug plates were inoculated, the first read was done following 24hr (72 hr for *Mycobacterium*) of incubation. Each drug dilution was inspected for growth of individual colonies. The results were recorded and the plates were re-incubated for another 24 hr (72 hr for *Mycobacterium*). At that time the final results were recorded and the MPC was determined by the lowest dilution of drug to have no growth of individual colonies. If the results were difficult to read, the plate in question was sub-cultured onto a fresh agar plate containing the same drug concentration and incubated, as described, for another 24 hr (72 hr for *Mycobacterium*).

2.4 Kill Curves

Fresh organism was sub-cultured onto 5 TSA plates for *S. pneumoniae* and one chocolate agar plate for *H. influenzae*. Plates were then incubated for 24hr as described. Organisms were transferred from the plates to 5 ml of broth (THB for *S. pneumoniae*,

BHI plus 5% fildes for *H. influenzae*) and then incubated for 2 hr as described. Growth of the bacterial suspension was checked with the spectrophotometer. An O.D. reading ≈ 1.7 at 600 nm approximates 1.0 x 10^9 cfu/ml. The 5 ml suspension was serially diluted by taking out 700 µl and adding it to 7 ml of fresh broth. This was done in triplicate. Seven hundred µl was removed from the last tube and discarded. This resulted in a four tube dilution series with inocula approximating 10^9, 10^8, 10^7 and 10^6 cfu/ml. At this point the antimicrobial agent was added at either the MIC or MPC drug concentration. The four dilutions were vortexed and then incubated for 24 hr as described. Samples from each of the four dilutions were taken at 0, 0.5, 1, 2, 4, 6, 12 and 24hr. Samples consisted of 100 µl aliquots which were diluted via 1:10 dilutions and then plated in triplicate (100 µl added to each agar plate). The samples were diluted so that viable counts had a countable number of colonies (20-200). The plates were then incubated for 24 hr as described. The 24 hr sample was taken, and the plates which had been inoculated with the 0 to 12 hr samples were read to determine the colony counts. The 24 hr sample was read following 24 hr of incubation as described.

3.0 Results

3.1 MIC Results for Ocular Isolates

The goal of this project was to examine the *in vitro* potency of five fluoroquinolones, using different methods, to eradicate organisms associated with ocular infections. For my experiments, two of the most common Gram-positive ocular pathogens (*S. pneumoniae, S. aureus*), two of the most common Gram-negative ocular pathogens (*H. influenzae, P. aeruginosa*) and two atypical ocular pathogens (*M. chelonae, M. fortuitum*) were selected for testing. Because ocular specimens are not routinely collected, the number of clinical isolates for each organism was rather limited. Consequently, the following number of isolates were collected and tested: 38 *S. pneumoniae*, 8 *S. aureus*, 22 *P. aeruginosa*, 31 *H. influenzae*, 5 *M. fortuitum* and 4 *M. chelonae*.

The MIC results were summarized by calculating the MIC_{50}, the modal value, and the MIC_{90}. The MIC_{50} represents the concentration of drug required to inhibit 50% of the isolates tested. Likewise, the MIC_{90} represents the concentration of drug required

to inhibit 90% of the isolates tested. The results are displayed by showing the MIC distribution and then by calculating the MIC_{50} and MIC_{90} values for each organism. For organisms where fewer than 10 isolates were tested the MIC_{50} and MIC_{90} values were not calculated.

It was expected that the new fluoroquinolones (moxifloxacin, gatifloxacin) would show more *in vitro* potency against the Gram-positive organisms than the older fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin). The results supported this hypothesis. For *S. pneumoniae,* the MIC distribution showed the newer fluoroquinolones furthest to the left at the lower drug concentrations while the older fluoroquinolones shifted considerably to the right towards the higher drug concentrations (Table 3.1.1). For moxifloxacin, the majority of isolates had values of either 0.063 or 0.125 μg/ml. In this case both the MIC_{50} and MIC_{90} were identical with a value of 0.125 μg/ml. For gatifloxacin the results were slightly higher than moxifloxacin with the majority of isolates having MIC values of either 0.125 or 0.25 μg/ml. Again, both the MIC_{50} and MIC_{90} were the same, having a value of 0.25 μg/ml (one dilution higher than moxifloxacin). The MIC values for levofloxacin were between 4 and 8-fold higher than moxifloxacin and gatifloxacin. Roughly half of the *S. pneumoniae* isolates tested against levofloxacin had MIC values of either 0.5 or 1 μg/ml. In this case there was a one dilution difference between the MIC_{50} (0.5 μg/ml) and the MIC_{90} which was 1 μg/ml. The results for ofloxacin and ciprofloxacin were very similar. They had the highest MIC values of the five fluoroquinolones tested. Ofloxacin, however, had a higher number of isolates with values of 2 μg/ml. This difference was reflected in the MIC_{50}, which was 2 μg/ml for ofloxacin and 1 μg/ml for ciprofloxacin. The MIC_{90} values were identical at 2 μg/ml. Overall, for *S. pneumoniae*, the rank order of potency was moxifloxacin > gatifloxacin > levofloxacin > ciprofloxacin > ofloxacin.

A similar trend was observed with *S. aureus,* except there was not as large a difference between the older and newer fluoroquinolones. The MIC distribution showed a gradual increase in MIC values as the progression from newer to older fluoroquinolones was made (Table 3.1.2). Moxifloxacin had MIC values ranging from 0.016 to 0.031 μg/ml, with 63% of isolates at the higher dilution. Gatifloxacin had MIC

values which were 1-2 dilutions higher than those for moxifloxacin. The values ranged from 0.063 – 0.125 μg/ml with, 75% of isolates having the lower value as their MIC. Levofloxacin was only slightly less potent than gatifloxacin with 7 out of 8 isolates having an MIC of 0.125 μg/ml. The results for ofloxacin and ciprofloxacin were, again, similar. Both fluoroquinolones had MIC_{90} values of 0.25 μg/ml. For ciprofloxacin two isolates had MIC values of 0.125 μg/ml. As a result, the rank of potency between the five drugs for *S. aureus* was moxifloxacin > gatifloxacin > levofloxacin > ciprofloxacin > ofloxacin.

For the Gram-negative organisms it was expected that the new fluoroquinolones would maintain the same activity as the older fluoroquinolones; this was not the case. For *H. influenzae*, the distribution had a much different appearance than that of the Gram-positives (Table 3.1.3). While the ranges differed somewhat between the five fluoroquinolones the MIC_{50} and MIC_{90} values were essentially identical. There was a large range of MIC values for moxifloxacin, from 0.004 – 0.063 μg/ml. The MIC_{90} value (0.031 μg/ml) was only one dilution higher than the MIC_{50} value (0.016 μg/ml). Gatifloxacin had a slightly narrower range with MIC values of 0.008 – 0.031 μg/ml. The MIC_{50} and MIC_{90} values, however, were identical to that of moxifloxacin. The range of MIC values for levofloxacin was 0.002 – 0.031 μg/ml. With both an MIC_{50} and MIC_{90} 0.016 μg/ml, the values for levofloxacin were lower than that of moxifloxacin and gatifloxacin. It was expected that results for ofloxacin would resemble that of ciprofloxacin but this was not the case. The MIC distribution for ofloxacin was similar to that of moxifloxacin. The MIC values ranged from 0.008 – 0.063 μg/ml. The MIC_{50} and MIC_{90} values were 0.016 and 0.031 μg/ml respectively. Ciprofloxacin was the most potent of the five fluoroquinolones. The MICs ranged from 0.001 – 0.016 μg/ml. The MIC_{50} value was lower than the other fluoroquinolones by

		MIC Distribution (ug/ml)									
Drug	***n***	**0.031**	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**MIC_{50}[a]**	**MIC_{90}[a]**
Moxifloxacin	38	1	15	21	1					0.125	0.125
Gatifloxacin	38		1	15	20	2				0.25	0.25
Levofloxacin	38					20	18			0.5	1
Ofloxacin	38						10	27	1	2	2
Ciprofloxacin	38					6	21	9	2	1	2

Table 3.1.1: **Summary of the *in vitro* potency of the five fluoroquinolones tested against ocular *S. pneumoniae* isolates**.
[a] The drug concentration inhibiting 50% and 90% of isolates tested respectively.
MIC = the Minimum Inhibitory Concentration.

		MIC Distribution (ug/ml)					
Drug	***n***	**0.016**	**0.031**	**0.063**	**0.125**	**0.25**	**0.5**
Moxifloxacin	8	3	5				
Gatifloxacin	8			6	2		
Levofloxacin	8				7	1	
Ofloxacin	8					7	1
Ciprofloxacin	8				2	5	1

Table 3.1.2: **Summary of the *in vitro* potency of the five fluoroquinolones tested against ocular *S. aureus* isolates**.
MIC = the Minimum Inhibitory Concentration. The MIC_{50} and MIC_{90} values were not calculated as the number of isolates tested was less than 10.

		MIC Distribution (ug/ml)								
Drug	***n***	**0.001**	**0.002**	**0.004**	**0.008**	**0.016**	**0.031**	**0.063**	**MIC_{50}[a]**	**MIC_{90}[a]**
Moxifloxacin	31			7	7	12	4	1	0.016	0.031
Gatifloxacin	31				15	9	7		0.016	0.031
Levofloxacin	31		1	4	9	15	2		0.016	0.016
Ofloxacin	31				1	21	8	1	0.016	0.031
Ciprofloxacin	31	1	4	5	14	7			0.008	0.016

Table 3.1.3: **Summary of the *in vitro* potency of the five fluoroquinolones tested against ocular *H. influenzae* isolates**.
[a] The drug concentration inhibiting 50% and 90% of isolates tested respectively.
MIC = the Minimum Inhibitory Concentration.

		MIC Distribution (ug/ml)											
Drug	***n***	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**MIC_{50}[a]**	**MIC_{90}[a]**
Moxifloxacin	21			1	2	10	2	4	2		1	1	8
Gatifloxacin	21			3	7	5	3	2	1			1	4
Levofloxacin	21		1	3	5	6	4	2			1	1	4
Ofloxacin	21				3	7	5	4	2		1	2	8
Ciprofloxacin	21	4	4	6	5	1	1			1		0.25	1

Table 3.1.4: **Summary of the *in vitro* potency of the five fluoroquinolones tested against ocular *P. aeruginosa* isolates**.
[a] The drug concentration inhibiting 50% and 90% of isolates tested respectively.
MIC = the Minimum Inhibitory Concentration.

		MIC Distribution (ug/ml)						
Drug	***n***	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
Moxifloxacin	4			1		2	1	
Gatifloxacin	4	1				1	2	
Levofloxacin	4		1				1	2
Ofloxacin	4					1		3
Ciprofloxacin	4			1		1	2	

Table 3.1.5: **Summary of the *in vitro* potency of the five fluoroquinolones tested against systemic *M. chelonae* isolates**.
MIC = the Minimum Inhibitory Concentration. The MIC_{50} and MIC_{90} values were not calculated as the number of isolates tested was less than 10.

		MIC Distribution (ug/ml)					
Drug	***n***	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**
Moxifloxacin	5	2	2	1			
Gatifloxacin	5	1	2	1	1		
Levofloxacin	5			1	3	1	
Ofloxacin	5				2	2	1
Ciprofloxacin	5		3	1	1		

Table 3.1.6: **Summary of the *in vitro* potency of the five fluoroquinolones tested against systemic *M. fortuitum* isolates**.
MIC = the Minimum Inhibitory Concentration. The MIC_{50} and MIC_{90} values were not calculated as the number of isolates tested was less than 10.

Organism	Drug	Susceptible	Intermediate	Resistant
S. aureus	Ciprofloxacin	≤1	2	≥4
	Levofloxacin	≤2	4	≥8
	Gatifloxacin	≤2	4	≥8
S. pneumoniae	Gatifloxacin	≤1	2	≥4
	Levofloxacin	≤2	4	≥8
	Moxifloxacin	≤1	2	≥4
H. influenzae	Ciprofloxacin	≤1		
	Gatifloxacin	≤1		
	Levofloxacin	≤2		
	Moxifloxacin	≤1		
	Ofloxacin	≤2		
P. aeruginosa	Ciprofloxacin	≤1	2	≥4
	Levofloxacin	≤2	4	≥8
	Ofloxacin	≤2	4	≥8
	Gatifloxacin	≤2	4	≥8

Figure 3.1.7: Fluoroquinolone breakpoints (80). Intermediate and resistant values were not available for *H. influenzae.*

one dilution, with a value of 0.008 μg/ml. The MIC_{90} value was 0.016 μg/ml, which was the same as for levofloxacin. Despite the minor differences, all five fluoroquinolones were potent *in vitro* with 100% of isolates inhibited by ≤ 0.063 μg/ml of any drug. The rank order of potency was ciprofloxacin = levofloxacin > ofloxacin = gatifloxacin = moxifloxacin.

The MIC distribution for *P. aeruginosa* showed a large MIC range for each of the quinolones (Table 3.1.4). For moxifloxacin the range of MIC values was 0.25 – 32 μg/ml. The largest number of isolates (47%) had an MIC value of 1 μg/ml (MIC_{50}) whereas the MIC_{90} value was 8-fold higher at 8 μg/ml. For gatifloxacin the MIC range was 0.25 – 8 μg/ml. While the largest number of isolates (33%) had an MIC value 0.5 μg/ml, one dilution lower than for moxifloxacin, the MIC_{50} values were the same at 1 μg/ml. The MIC_{90} however, was one dilution lower at 4 μg/ml. Levofloxacin had one of the widest ranges with MIC values from 0.125 – 32 μg/ml. Like gatifloxacin, levofloxacin had MIC_{50} and MIC_{90} values of 1 and 4 μg/ml respectively. Surprisingly ofloxacin had the lowest potency. The MIC values ranged from 0.5 – 32 μg/ml. While the majority of isolates had MICs of 1 μg/ml the MIC_{50} was 2 μg/ml. The MIC_{90} was the same as for moxifloxacin at 8 μg/ml. Ciprofloxacin had the most potent *in vitro* activity with MIC values 4 – 8-fold lower than that of the other fluoroquinolones. The MIC range was 0.063 – 16 μg/ml and MIC_{50} and MIC_{90} values of 0.25 and 1 μg/ml respectively. While ciprofloxacin's *in vitro* potency was significantly better than the others, the four other fluoroquinolones had quite similar MIC values. The rank order of potency for *P. aeruginosa* was ciprofloxacin > levofloxacin = gatifloxacin > ofloxacin = moxifloxacin.

For the atypical mycobacteria, it was expected that the new fluoroquinolones would have lower MICs compared to the older fluoroquinolones. This turned out to be the case, except that ciprofloxacin showed equivalent activity as shown in tables 3.1.5 and 3.1.6. Due to the low number of isolates only loose observations can be made. For *M. chelonae*, moxifloxacin, gatifloxacin and ciprofloxacin appeared to have similar potency with the majority of the isolates having MIC values of 4 – 8 μg/ml. Both levofloxacin and ofloxacin seemed to have slightly less *in vitro* potency with the

majority of the isolates having MIC values ≥ 16 μg/ml. The resulting rank order of potency was moxifloxacin = gatifloxacin = ciprofloxacin > levofloxacin = ofloxacin.

The MIC trends for *M. fortuitum* were similar to that of *M. chelonae*, except that the MIC values seemed to be lower. For moxifloxacin, gatifloxacin and ciprofloxacin, the majority of the MIC values were between 0.125 and 0.25 μg/ml. Both levofloxacin and ofloxacin had MIC values between 1 and 2 μg/ml which was approximately 2-fold higher than the other fluoroquinolones. The rank order of potency for *M. fortuitum* was moxifloxacin = gatifloxacin = ciprofloxacin > levofloxacin = ofloxacin.

3.2 MPC Results for Ocular Isolates

The MPC results showed the same trends as did the MIC results. The number of ocular isolates tested using MPC fluctuated from test to test. This is because, at least for the fastidious organisms like *S. pneumoniae* and *H. influenzae*, it was difficult to get the organisms to grow to an inoculum size ≥ 10^9 cfu/ml as required for the MPC test. While all ocular isolates were tested, only the results from the experiments which attained the proper inoculum size were included in the summary. The results were summarized by calculating the MPC_{50} and MPC_{90}. The MPC_{50} represents the concentration of drug required to prevent the growth of first-step mutants for 50% of the isolates tested. MPC_{90} represents the concentration of drug required to inhibit the growth of first-step mutants for 90% of the bacterial isolates tested.

Based on previous observations from our laboratory it was anticipated that the MPC values would be higher than those from the MIC testing because it should be harder to inhibit a population of bacteria which, firstly, is larger than that used in MIC testing, and second, that may possess first-step resistance mutations. It was also expected that the rank order of potency established by MIC would be maintained for the MPC results. For the most part, both of these working assumptions were correct.

Starting with clinical eye isolates of *S. pneumoniae*, the newer fluoroquinolones showed four to eight times the potency compared to the older fluoroquinolones. The moxifloxacin MPC values ranged from 0.125 – 1 μg/ml (Table 3.2.1). The vast majority of isolates (56%) had MPC values of 0.5 μg/ml, which was the same as the MPC_{50}. The MPC_{90} was one dilution higher at 1 μg/ml. The results for gatifloxacin were similar. The range, however, was much narrower, with MPC values from 0.5 – 2

μg/ml. The MPC_{50} value was 1 μg/ml, one dilution higher than that of moxifloxacin. The MPC_{90} was also 1 μg/ml, the same as for moxifloxacin. The results for levofloxacin showed a slight shift to the right in the MPC distribution. The MPC values ranged from 1 – 4 μg/ml. The MPC_{50} value was 2 μg/ml while the MPC_{90} value was 2-fold higher at 4 μg/ml. The results for both ofloxacin and ciprofloxacin were identical except that the range for ciprofloxacin extended from 2 – 16 μg/ml as opposed to 2 – 8 μg/ml for ofloxacin. Both had MPC_{50} values of 4 μg/ml and MPC_{90} values of 8 μg/ml. The rank potency order was moxifloxacin = gatifloxacin > levofloxacin > ciprofloxacin = ofloxacin.

The *S. aureus* results, like *S. pneumoniae*, showed a gradual shift in the MPC distribution to the right with the progression of newer to older fluoroquinolones (Table 3.2.2). Moxifloxacin appeared to have the most *in vitro* potency with lower MPC values. All of the MPC values were 0.125 μg/ml. Gatifloxacin had MPC values of 0.25 μg/ml, one dilution greater than moxifloxacin and levofloxacin was 2-fold less active than gatifloxacin having MPC values of 0.5 μg/ml. The majority of isolates tested against ofloxacin had MPC values of 1 μg/ml. Ciprofloxacin was 2-fold less potent than ofloxacin with the majority of isolates having MPC values of 2 μg/ml. From the newest fluoroquinolone to the oldest, there was one dilution decrease in *in vitro* potency resulting in a potency rank order of moxifloxacin > gatifloxacin > levofloxacin > ofloxacin > ciprofloxacin.

All five fluoroquinolones were very effective *in vitro* against *H. influenzae*. For moxifloxacin, there was a range of MPC values from 0.063 – 0.5 μg/ml (Table 3.2.3). The majority (42%) of the isolates had MPC values of 0.5 μg/ml. The MPC_{50} was 0.25 μg/ml with the MPC_{90} one concentration higher at 0.5 μg/ml. The results for gatifloxacin were slightly lower with a range of MPC values from 0.063 – 0.25 μg/ml. Both the MPC_{50} and MPC_{90} values were 0.125 μg/ml. For levofloxacin, the MPC values were identical to that of gatifloxacin. Ofloxacin had the same MPC values as moxifloxacin with an MPC_{50} of 0.25 μg/ml and a MPC_{90} of 0.5μg/ml. The distribution was slightly different with values ranging from 0.125 - ≥ 1 μg/ml. Ciprofloxacin had the widest distribution with MPC values from 0.063 - ≥ 1 μg/ml. The MPC_{50} and

MPC_{90} values were 0.125 and 0.5 μg/ml respectively. The rank order of potency was gatifloxacin = levofloxacin > ciprofloxacin > moxifloxacin = ofloxacin.

Overall, the MPC values for *P. aeruginosa* were considerably higher than those observed for the other pathogens (Table 3.2.4). Moxifloxacin exhibited the least *in vitro* potency with an MPC distribution ranging from 8 - ≥ 64 μg/ml. The MPC_{50} value was 16 μg/ml while the MPC_{90} value was 2-fold higher at 32 μg/ml. Gatifloxacin had MPC values which were marginally lower and the MPC distribution ranged from 4 – 32 μg/ml. With the majority (52%) of the isolates having an MPC value of either 4 or 8 μg/ml, both the MPC_{50} and MPC_{90} values were 8 μg/ml. The MPC results for levofloxacin were the same as for gatifloxacin, both the MPC_{50} and MPC_{90} were 8 μg/ml. Ofloxacin had an MPC distribution of 8 - ≥ 64 μg/ml, similar to the results observed for moxifloxacin. Both the MPC_{50} and MPC_{90} values were 16 μg/ml. Ciprofloxacin exhibited the greatest *in vitro* potency with an MPC range of 1 - 16μg/ml; the MPC_{50} and MPC_{90} values were 2 μg/ml. Ciprofloxacin was between 4 and 16 times as potent as the other fluoroquinolones. The *in vitro* rank potency order was ciprofloxacin > gatifloxacin = levofloxacin > ofloxacin > moxifloxacin.

The results for the MPC testing of the 5 fluoroquinolones against atypical mycobacterial strains are summarized in tables 3.2.5 and 3.2.6. With regards to *M. chelonae*, there was a wide MPC distribution for all fluoroquinolones. For moxifloxacin, the MPC values ranged from 4 – 256 μg/ml with each isolate having a different MPC value. The MPC results for gatifloxacin showed a range from 32 – 256 μg/ml, however, all but one of the isolates had an MPC value of 32 μg/ml. Levofloxacin also had a wide MIC range with MPC values of 16 - ≥ 512 μg/ml. Ofloxacin had MPC values were only spread over three dilutions ranging from 64 – 256 μg/ml. Ciprofloxacin had a slightly narrower MPC distribution with values from 8 – 128 μg/ml. The rank of potency order was ciprofloxacin > moxifloxacin = gatifloxacin =ofloxacin>levofloxacin.

		MPC Distribution (ug/ml)									
Drug	***n***	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**MPC_{50}[a]**	**MPC_{90}[a]**
Moxifloxacin	33	4	2	19	7		1			0.5	1
Gatifloxacin	31			9	19	3				1	1
Levofloxacin	33				5	23	5			2	4
Ofloxacin	37					3	20	14		4	8
Ciprofloxacin	32					5	20	4	3	4	8

Table 3.2.1: **Summary of the *in vitro* potency as determined by MPC testing of five fluoroquinolones tested against ocular *S. pneumoniae* isolates**.

[a] The drug concentration inhibiting 50% and 90% of isolates tested respectively.

MPC = the Mutant Prevention Concentration.

		MPC Distribution (ug/ml)					
Drug	***n***	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**
Moxifloxacin	8	8					
Gatifloxacin	8		8				
Levofloxacin	8			8			
Ofloxacin	8				7	1	
Ciprofloxacin	8				3	4	1

Table 3.2.2: **Summary of the *in vitro* potency as determined by MPC testing of five fluoroquinolones tested against ocular *S. aureus* isolates**.

MPC = the Mutant Prevention Concentration. The MPC_{50} and MPC_{90} values were not calculated as the number of isolates tested was less than 10.

		MPC Distribution (ug/ml)						
Drug	***n***	**0.063**	**0.125**	**0.25**	**0.5**	**≥1**	**MPC_{50}[a]**	**MPC_{90}[a]**
Moxifloxacin	26	1	7	7	11		0.25	0.5
Gatifloxacin	26	9	16	1			0.125	0.125
Levofloxacin	25	2	22	1		1	0.125	0.125
Ofloxacin	26		2	18	5	1	0.25	0.5
Ciprofloxacin	26	5	13		6	2	0.125	0.5

Table 3.2.3: **Summary of the *in vitro* potency as determined by MPC testing of five fluoroquinolones tested against ocular *H. influenzae* isolates**.

[a] The drug concentration inhibiting 50% and 90% of isolates tested respectively.

MPC = the Mutant Prevention Concentration.

		MPC Distribution (ug/ml)								
Drug	***n***	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**MPC_{50}[a]**	**MPC_{90}[a]**
Moxifloxacin	21				1	10	8	2	16	32
Gatifloxacin	21			8	11		2		8	8
Levofloxacin	21			4	15		2		8	8
Ofloxacin	21				5	14		2	16	16
Ciprofloxacin	21	3	15	1	1	2			2	2

Table 3.2.4: **Summary of the *in vitro* potency as determined by MPC testing of five fluoroquinolones tested against ocular *P. aeruginosa* isolates**.

[a] The drug concentration inhibiting 50% and 90% of isolates tested respectively.

MPC = the Mutant Prevention Concentration.

		MPC Distribution (ug/ml)							
Drug	***n***	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**≥512**
Moxifloxacin	4	1	1			1		1	
Gatifloxacin	4				3			1	
Levofloxacin	4			2		1			1
Ofloxacin	4					1	1	2	
Ciprofloxacin	4		1		2		1		

Table 3.2.5: **Summary of the *in vitro* potency as determined by MPC testing of five fluoroquinolones tested against systemic *M. chelonae* isolates**.

MPC = the Mutant Prevention Concentration. The MPC_{50} and MPC_{90} values were not calculated as the number of isolates tested was less than 10.

		MPC Distribution (ug/ml)				
Drug	***n***	**0.5**	**1**	**2**	**4**	**8**
Moxifloxacin	5	2	1	2		
Gatifloxacin	5	1	3	1		
Levofloxacin	5				4	1
Ofloxacin	5				2	3
Ciprofloxacin	5		2	3		

Table 3.2.6: **Summary of the *in vitro* potency as determined by MPC testing of five fluoroquinolones tested against systemic *M. fortuitum* isolates**.
MPC = the Mutant Prevention Concentration. The MPC_{50} and MPC_{90} values were not calculated as the number of isolates tested was less than 10.

All five fluoroquinolones had much lower MPCs against *M. fortuitum* than against *M. chelonae*. The MPC distribution for moxifloxacin and gatifloxacin extended from 0.5 to 2 μg/ml. Levofloxacin and ofloxacin had higher MPC values ranging from 4 – 8 μg/ml . Ciprofloxacin had similar values to moxifloxacin and gatifloxacin with an MPC distribution of 1 – 2 μg/ml. The rank of potency order was moxifloxacin = gatifloxacin = ciprofloxacin > ofloxacin = levofloxacin.

3.3 Kill Curve Results for Ocular Isolates

Kill curve experiments were conducted to examine the bactericidal activities of the five fluoroquinolones against bacterial inocula ranging from 10^6 – 10^9 cfu/ml. Current literature presents kill curve data using inoculum sizes of 10^5 - 10^6cfu/ml. However, because actual bacterial loads of an ocular infection may reach 10^6 cfu or 10^7 cfu it was decided that the tests should incorporate a range of inoculum sizes (78). Because *S. pneumoniae* and *H. influenzae* are some of the most common pathogens of their respective Gram-positive and Gram-negative classes, they were selected as the most representative organisms to be tested. The sample size was initially set at seven, as seen in the *S. pneumoniae* MPC kill curves for gatifloxacin and moxifloxacin (Tables 3.3.1.3 and 3.3.1.7), however, due to the fastidious nature of this organism, a smaller sample size of four isolates was much more feasible given the time lines available for this project. That same number of organisms was used for testing *H. influenzae*. The drug concentrations used for these experiments were set using two different sets of parameters. The first was using the pre-determined MIC value for each isolate. Because the MIC value is such a globally used measurement, it was thought that it would be important to examine the pharmacodynamic properties for this set of drug concentrations. The second parameter used was the pre-determined MPC values for each isolate. Because it has been argued (65) that the MPC model provides a much more realistic drug concentration needed for eradication of a heterogeneous bacterial population (including both susceptible and first-step resistant organisms), it was important to have pharmacodynamic results at the MPC drug concentration to compare to the traditional MIC measurements.

3.3.1 *S. pneumoniae*

The *S. pneumoniae* kill results for moxifloxacin at the MIC are summarized in tables 3.3.1.1, and 3.3.1.2 (Fig. 3.3.1.3 – 3.3.1.8). At six hours there was a > 90 % reduction in viable cells at 10^7 cfu/ml, which is equivalent to a > 2 log reduction (Fig. 3.3.1.3). It was not until 12 hours that we see this same reduction with the other inocula. At 24 hours the reduction in viable cells is more pronounced for the 10^8 and 10^9 cfu/ml inocula, however, re-growth was seen at 10^6 and 10^7 cfu/ml. At the MPC drug concentration, killing begins much sooner as shown in table 3.3.1.4 (Fig. 3.3.1.4 – 3.3.1.8). As early as two hours after drug exposure, there was a 91% reduction in viable cells at the 10^7 cfu/ml inoculum. By four hours, three of the lower inocula reached a > 98 % viable cell reduction while it was not until 12 hours that moxifloxacin achieved this with the 10^9 cfu/ml inoculum. Unlike the results for the MIC kill curves, the killing at the MPC continued right up until 24 hours at which time all inocula were reduced by 100%.

The results for gatifloxacin at the MIC drug concentration are shown in table 3.3.1.6 (Fig. 3.3.1.3 – 3.3.1.8). At 10^6 cfu/ml, re-growth was observed immediately and continued throughout the 24 hours except for the slight viable cell reduction that occurred between the 0.5 and 1 hour interval. At 10^7 cfu/ml, a slight viable cell reduction of 40% was achieved by 6 hours, however, it was followed by re-growth which continued to the end of the test period. The 10^8 cfu/ml inoculum began with growth that continued until the 6 hour interval. At 12 hours a reduction in cell number was noticed. This trend continued, and by 24 hours a 83% reduction in viable cells had occurred. At 10^9 cfu/ml there was a decrease in viable cells immediately following drug exposure which continued until it reached a ≥ 99.99% (>3 log reduction Fig. 3.3.1.7) reduction by 24 hours. At the MPC drug concentration, an overall reduction in viable cells was observed much sooner than with the MIC (Table 3.3.1.8) For the lower three inocula there was a ≥ 98% reduction by 4 hours which reached 100% by 24 hours. As with the moxifloxacin MPC kill results, the 10^9 cfu/ml inoculum had a reduction in viable cells that occurred at a slower rate than that seen with the lower inocula. By 12 hours, the reduction in viable cells was ≥ 99%.

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.003	-0.2	-0.4	-0.81	-1.14	-1.7	-1.3
10^7	0.057	-0.23	-0.44	-1.15	-1.46	-2.08	-0.69
10^8	0.07	-0.04	-0.12	-0.097	-0.34	-1.07	-1.56
10^9	0.005	-0.025	-0.09	-0.24	-0.49	-2.12	-4.24

Table 3.3.1.1: The average $\log_{10}$ reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to moxifloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.063 – 0.125 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.48	-22.81	-29.18	-74.71	-84.81	-95.8	-92.6
10^7	13.86	-25.64	-68.58	-77.9	-96.49	-99.14	-62.31
10^8	16.2	7.8	34.43	20.21	-48.44	-90.43	-95.48
10^9	0.67	-4.66	-15.88	-40.44	-67.19	-99.23	-99.99

Table 3.3.1.2: The average percentage reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to moxifloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.063 – 0.125 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.13	-0.38	-1.25	-2.04	-3.25	-3.98	-5.08
10^7	-0.019	-0.51	-1.28	-2.1	-3.08	-3.98	-5.12
10^8	-0.12	-0.32	-1.11	-2.3	-2.98	-3.63	-5.82
10^9	-0.074	-0.071	-0.54	-0.94	-1.36	-3.51	-6.44

Table 3.3.1.3: The average $\log_{10}$ reduction in viable cells of 7 clinical *S. pneumoniae* isolates exposed to moxifloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 7 isolates = 0.25 – 0.5 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-19.98	-55.01	-89.69	-98.4	-99.66	-99.98	-100
10^7	-27.79	-64.24	-91.29	-99.1	-98.4	-99.99	-100
10^8	-18.14	-56.44	-87.83	-99.16	-99.78	-99.82	-100
10^9	-14.94	-24.22	-53.27	-77.78	-88.72	-99.55	-100

Table 3.3.1.4: The average percentage reduction in viable cells of 7 clinical *S. pneumoniae* isolates exposed to moxifloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 7 isolates = 0.25 – 0.5 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.03	-0.033	-0.058	-0.19	0.66	1.27	0.32
10^7	-0.03	-0.05	-0.21	-0.28	-0.35	0.38	-0.07
10^8	0.07	0.055	0.21	0.22	0.048	-0.84	-1.8
10^9	-0.83	-0.83	-0.83	-1.06	-1.08	-1.8	-3.85

Table 3.3.1.5: The average $\log_{10}$ reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to gatifloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.125 – 0.25 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	7.49	-1.19	13.86	36.76	78.25	94.32	50.81
10^7	-9.38	-0.038	-28.87	-34.13	-39.26	52.73	15.32
10^8	15.23	10.79	37.9	38.13	8.01	-33	-83.33
10^9	-85.07	-85.33	-85.22	-91.3	-91.59	-98.42	-99.99

Table 3.3.1.6: The average percentage reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to gatifloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.125 – 0.25 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.12	-0.28	-1	-2.16	-2.86	-4.39	-5.75
10^7	-0.21	-0.46	-1.23	-2.5	-3.3	-5.56	-6.84
10^8	-0.12	-0.36	-0.9	-2.23	-2.78	-3.46	-5.79
10^9	-0.004	-0.12	-0.49	-0.6	-1.05	-3.28	-7.77

Table 3.3.1.7: The average $\log_{10}$ reduction in viable cells of 7 clinical *S. pneumoniae* isolates exposed to gatifloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 7 isolates = 0.5 – 2 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-13.07	-32.07	-80.51	-98.05	-99.53	-99.97	-100
10^7	-20.43	-39.47	-84.72	-99.56	-99.86	-99.99	-100
10^8	-19.53	-43.93	-82.43	-98.77	-99.82	-99.75	-100
10^9	-0.47	-12.98	-31.9	-57.07	-80.41	-99.83	-99.99

Table 3.3.1.8: The average percentage reduction in viable cells of 7 clinical *S. pneumoniae* isolates exposed to gatifloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 7 isolates = 0.5 – 2 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.083	-0.05	-0.16	-0.71	-0.9	-1.8	-0.24
10^7	-0.073	-0.075	-0.26	-1.28	-1.63	-2.14	-1.57
10^8	0.015	-0.023	-0.23	-0.49	-0.58	-0.88	-2.09
10^9	-0.008	-0.015	-0.03	-0.22	-0.62	-2.59	-4.26

Table 3.3.1.9: The average log_{10} reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to levofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.5 – 1 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-16.79	-8.95	-15.13	-69.47	-71.59	-90.31	39.03
10^7	-11.45	-14.69	-38.98	-89.42	-95.29	-97.53	-97
10^8	3.47	-3.2	-34.93	-48.46	-46.19	-72.84	-94.63
10^9	2.75	-1.97	-6.36	-54.89	-74.59	-99.31	-99.99

Table 3.3.1.10: The average percentage reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to levofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.5 – 1 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.02	-0.115	-1.04	-1.77	-2.19	-3.72	-5.15
10^7	-0.025	-0.235	-1.07	-2.03	-2.83	-4.64	-6.37
10^8	-0.063	-0.22	-1.23	-2.14	-2.72	-3.19	-5.1
10^9	0.0125	-0.143	-0.263	-0.57	-1.46	-2.32	-5.58

Table 3.3.1.11: The average log_{10} reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to levofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 2 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-3.71	-18.91	-88.41	-95.55	-98.84	-99.97	-100
10^7	-16.08	-39.5	-88.5	-98.91	-99.84	-100	-100
10^8	-12.26	-34.31	-91.37	-99.11	-99.74	-99.94	-100
10^9	12.52	-27.06	-44.08	-67.31	-92.36	-99.23	-100

Table 3.3.1.12: The average percentage reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to levofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 2 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	0.07	-0.085	-0.31	-0.6	-1.21	-1.86	-2.29
10^7	-0.008	-0.035	-0.23	-0.89	-1.62	-3	-1.46
10^8	0.0025	-0.018	-0.47	-0.38	-0.5	-1.31	-1.82
10^9	-0.025	-0.058	-0.113	-0.53	-1.17	-3	-5.06

Table 3.3.1.13: The average $\log_{10}$ reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to ofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 1 – 2 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	15.23	-15.72	-44.32	-58.96	-76.43	-58.1	-99.2
10^7	-0.82	-7.25	-34.65	-68.53	-90.87	-99.75	-95.77
10^8	1.12	-2.72	-45	-45.37	-61.54	-93.07	-98.31
10^9	-5.4	-12.05	-21.96	-60.02	-81.69	-98.97	-100

Table 3.3.1.14: The average percentage reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to ofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 1 – 2 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.12	-0.49	-1.21	-1.96	-3.11	-4.22	-5.31
10^7	-0.163	-0.59	-1.4	-2.53	-3.4	-4.57	-6.29
10^8	-0.128	-0.6	-1.7	-2.4	-3.21	-4.4	-5.83
10^9	-0.078	-0.36	-0.6	-1.15	-1.8	-3.74	-6.33

Table 3.3.1.15: The average $\log_{10}$ reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to ofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 4 - 8 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-6.25	-56.78	-85.28	-96.23	-99.78	-99.99	-100
10^7	-12.85	-59.88	-87.5	-99.58	-99.92	-100	-100
10^8	-9.15	-60.02	-67.11	-98.09	-99.02	-99.77	-100
10^9	-4.99	-50	-57.75	-78.52	-94.35	-99.91	-100

Table 3.3.1.16: The average percentage reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to ofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 4 – 8 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	0.103	0.015	-0.105	-0.413	-0.9	-0.93	1.11
10^7	-0.03	0.01	-0.125	-0.98	-1.33	-0.88	0.82
10^8	0.013	0.025	-0.06	-0.26	-0.19	-0.57	-2.01
10^9	0.043	0.06	-0.11	-0.52	-0.85	-2.54	-4.84

Table 3.3.1.17: The average $\log_{10}$ reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to ciprofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 1 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	31.88	3.52	-7.05	-26.99	-41.93	-87.9	91.34
10^7	-6.44	2.63	-17.16	-84.57	-94.34	-37.25	85.62
10^8	3.04	5.8	-10.66	-34.43	-32.28	-70.7	-98.79
10^9	7.81	13.86	-21.37	-50.79	-83.67	-99.36	-100

Table 3.3.1.18: The average percentage reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to ciprofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 1 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	0.003	-0.28	-1.02	-1.33	-1.9	-3.55	-4.26
10^7	-0.14	-0.37	-1.25	-2.19	-2.78	-4.11	-5.16
10^8	-0.11	-0.26	-1.18	-1.77	-2.65	-3.73	-3.99
10^9	-0.08	-0.16	-0.47	-0.89	-0.98	-1.83	-4.68

Table 3.3.1.19: The average log_{10} reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to ciprofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 2 - 4 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	4.32	-43.06	-87.16	-97.36	-95.58	-99.95	-99.99
10^7	-25.72	-51.41	-88.69	-99.12	-99.82	-99.99	-100
10^8	-20.78	-41.25	-86.71	-96.71	-99.34	-99.95	-99.99
10^9	-16.52	-29.98	-62.72	-85.46	-88.6	-94.39	-100

Table 3.3.1.20: The average percentage reduction in viable cells of 4 clinical *S. pneumoniae* isolates exposed to ciprofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 2 – 4 μg/ml

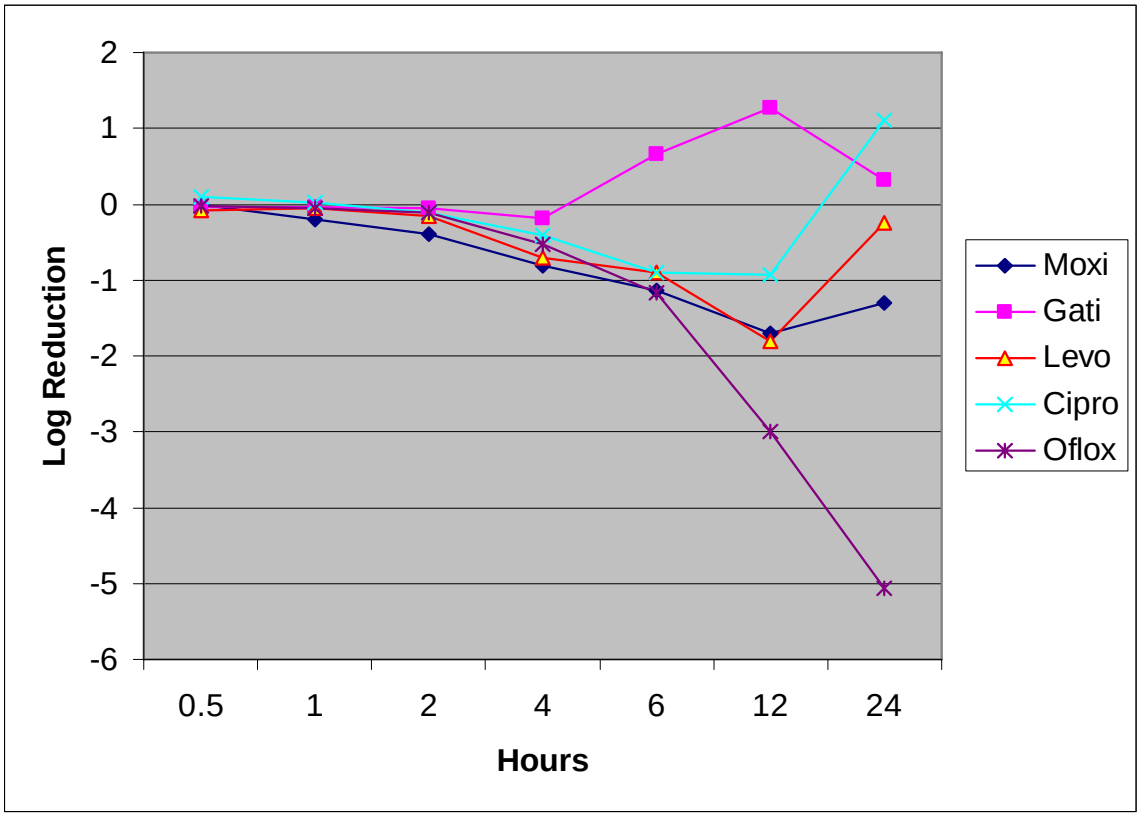

Figure 3.3.1.1: The Killing of *S. pneumoniae* ocular isolates (n = 4) at 10^6 cfu/ml following exposure to each of the five fluoroquinolones at the Minimum Inhibitory drug concentration.

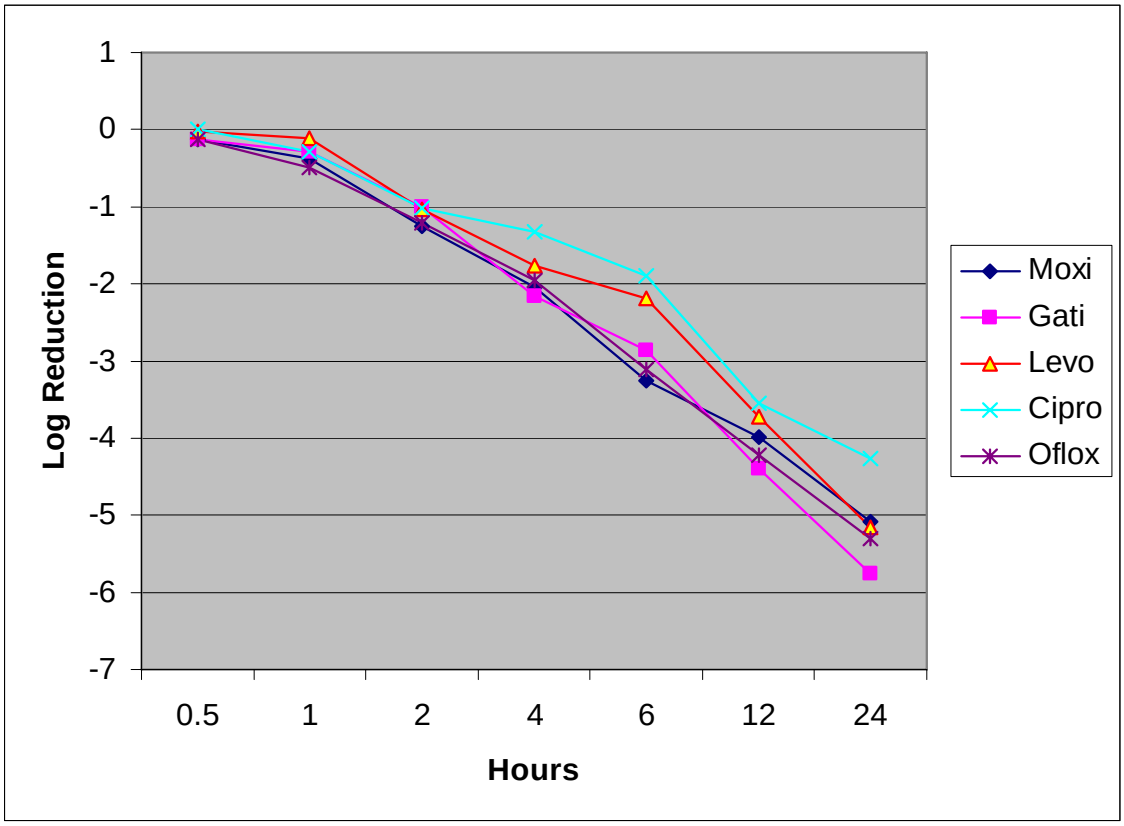

Figure 3.3.1.2: The Killing of *S. pneumoniae* ocular isolates (n = 4) at 10^6 cfu/ml following exposure to each of the five fluoroquinolones at the Mutant Prevention drug concentration.

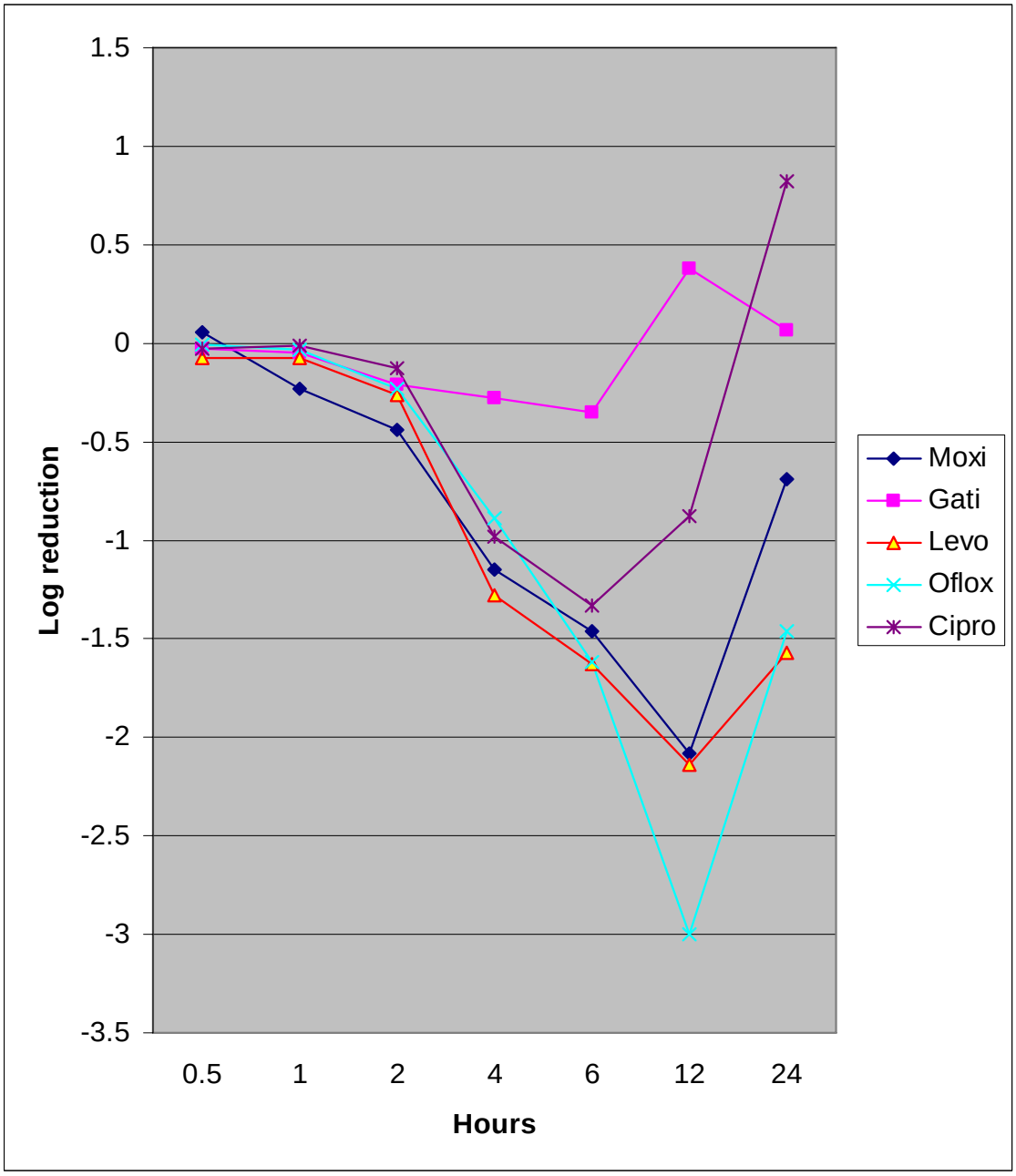

Figure 3.3.1.3: The Killing of *S. pneumoniae* ocular isolates (n = 4) at 10^7 cfu/ml following exposure to each of the five fluoroquinolones at the Minimum Inhibitory drug concentration.

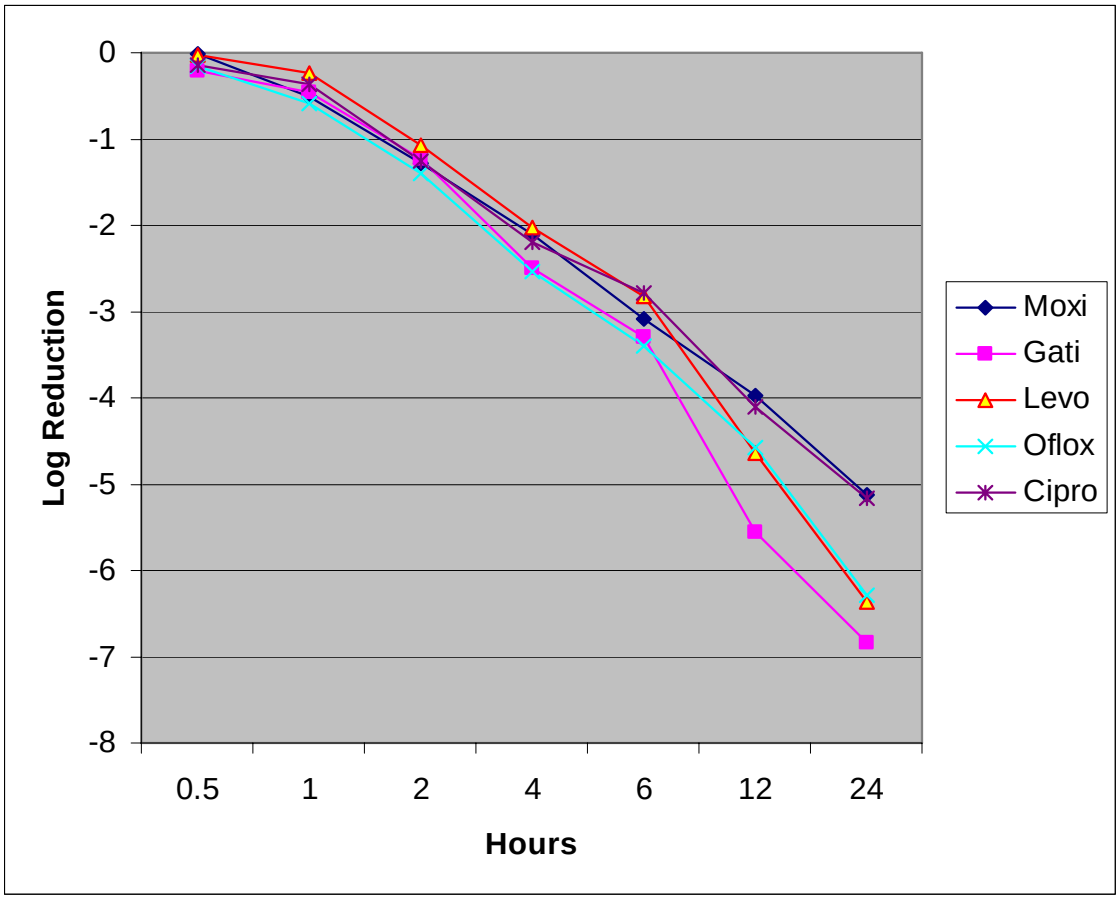

Figure 3.3.1.4: The Killing of *S. pneumoniae* ocular isolates (n = 4) at 10^7 cfu/ml following exposure to each of the five fluoroquinolones at the Mutant Prevention drug concentration.

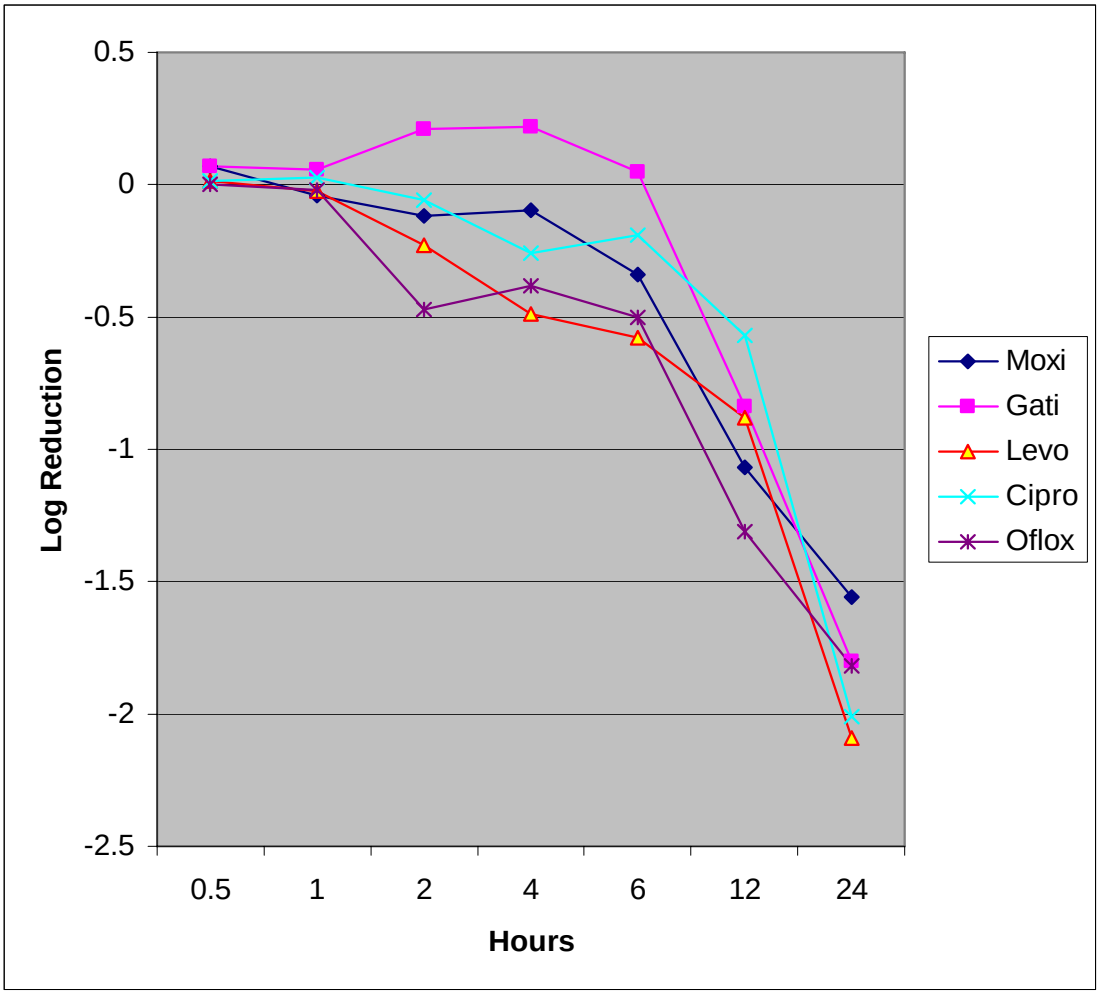

Figure 3.3.1.5: The Killing of *S. pneumoniae* ocular isolates (n = 4) at 10^8 cfu/ml following exposure to each of the five fluoroquinolones at the Minimum Inhibitory drug concentration.

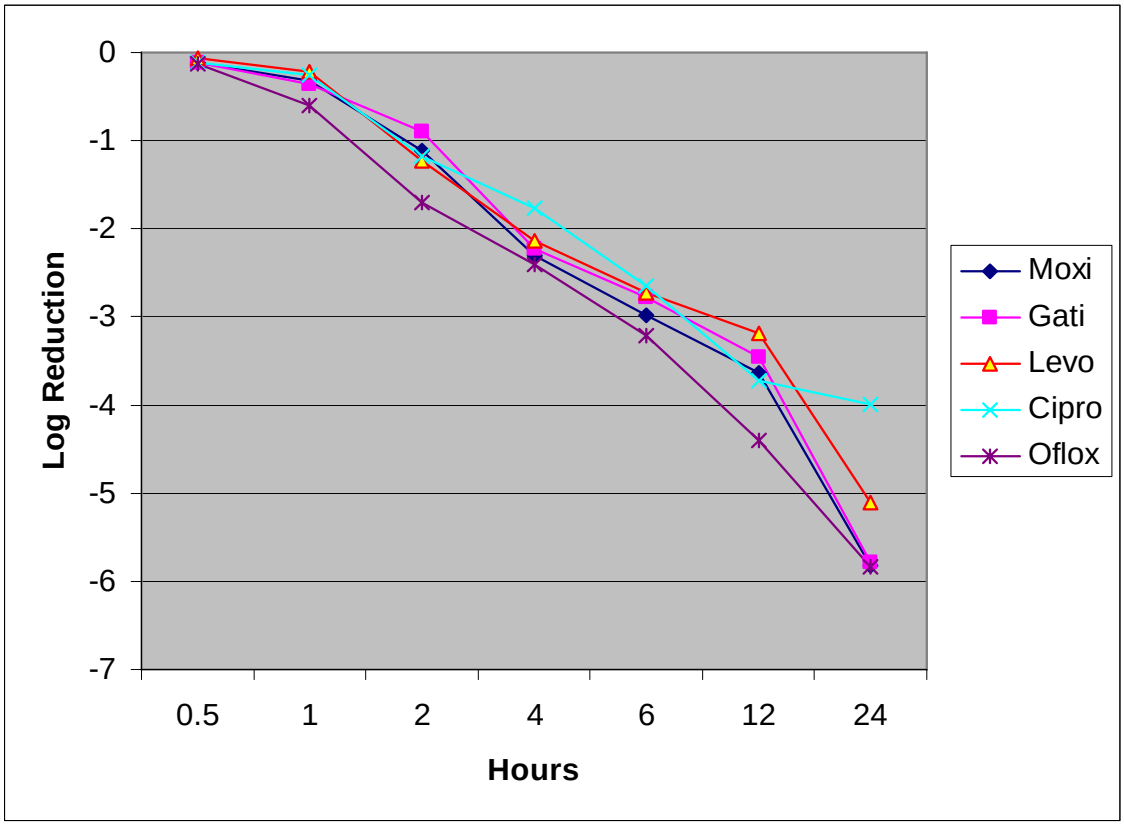

Figure 3.3.1.6: The Killing of *S. pneumoniae* ocular isolates (n = 4) at 10^8 cfu/ml following exposure to each of the five fluoroquinolones at the Mutant Prevention drug concentration.

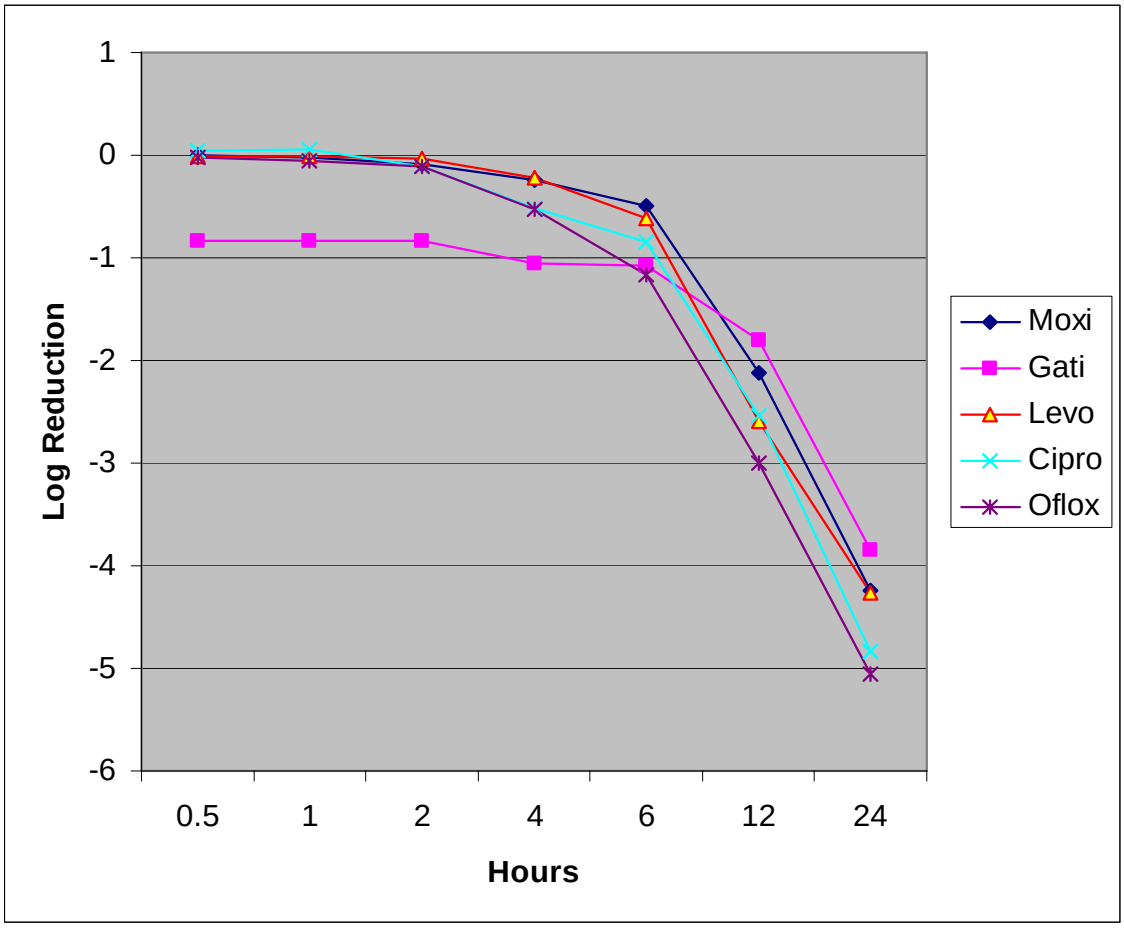

Figure 3.3.1.7: The Killing of *S. pneumoniae* ocular isolates (n = 4) at 10^9 cfu/ml following exposure to each of the five fluoroquinolones at the Minimum Inhibitory drug concentration.

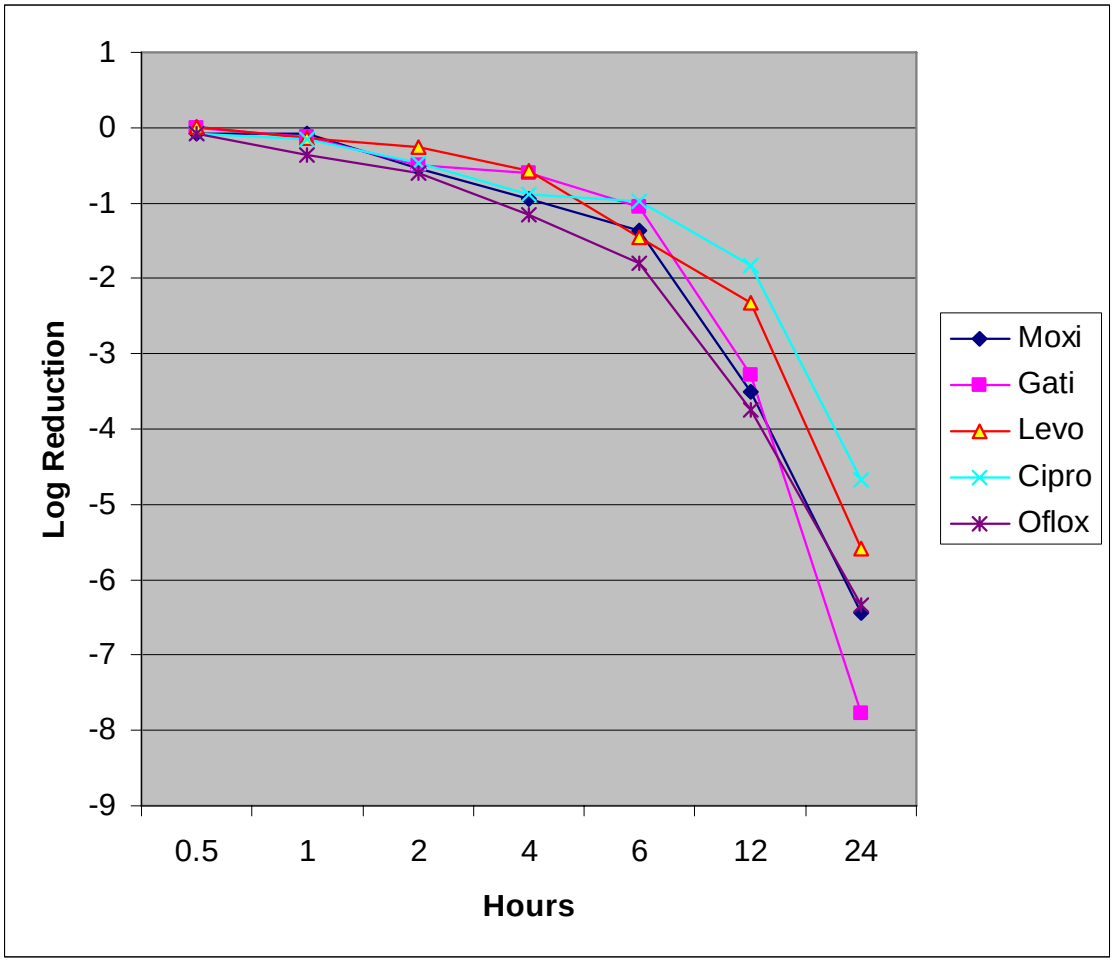

Figure 3.3.1.8: The Killing of *S. pneumoniae* ocular isolates (n = 4) at 10^9 cfu/ml following exposure to each of the five fluoroquinolones at the Mutant Prevention drug concentration.

At the MIC drug concentration for levofloxacin, there was a reduction in viable cells for the 10^6 and 10^7 cfu/ml inocula which peaked at 12 hours with a ≥ 90% (> 1.5 log) reduction (Table 3.3.1.10, Fig. 3.3.1.1 – 3.3.1.3). This was followed by re-growth at the 24 hour interval. The 10^8 cfu/ml inoculum showed a 95% reduction of viable cells after 24 hours of drug exposure. The 10^9 cfu/ml inoculum was reduced faster with a ≥ 99% reduction being achieved by 12 hours of drug exposure. At the MPC the three lower inocula had viable cells reduced by ≥ 95% at 4 hours and 100% by 24 hours of drug exposure (Table 3.3.1.12). The largest inoculum had a 92% reduction in viable cells by 6 hours and 100% by 24 hours.

With ofloxacin at the MIC drug concentration, significant viable cell reduction did not occur until 12 hours of drug exposure for the higher three inocula. (Table 3.3.1.14, Fig. 3.3.1.1 – 3.3.1.8). At 12 hours the 10^6 cfu/ml inoculum had a reduction in viable cells of 58%. By 24 hours, however, all inocula had been reduced by at least 95%. At the MPC, the results were similar to the previous three fluoroquinolones (Table 3.3.1.16). For the lower three inocula there was a significant reduction in viable cell by 4 hours (> 96%). The 10^9 cfu/ml inoculum had a 94% viable cell reduction by 6 hours and all inocula had a 100% reduction in viable cells after 24 hours of drug exposure.

With the exception of the 10^7 cfu/ml inoculum there was no reduction observed until 2 hours after exposure to ciprofloxacin at the MIC drug concentration (Table 3.3.1.18, Fig. 3.3.1.1 – 3.3.1.8). For the 10^6 cfu/ml inoculum, the reduction peaked at 88% at the 12 hour interval. Following that, there was re-growth. The results for the 10^7 cfu/ml inoculum were similar except the reduction in viable cells at 6 hours of drug exposure peaked at 94%. The 10^8 cfu/ml inoculum reached a 32% reduction after 6 hours of drug exposure and peaked with a 99% reduction of viable cells by 24 hours. At 6 hours the 10^9 cfu/ml inoculum had been reduced by 84%, and at 24 hours, 100%. At the MPC there was a viable cell reduction of ≥ 85% in all inocula by 4 hours (Table 3.3.1.20). By 24 hours all inocula had been reduced by ≥ 99.99%.

3.3.2 *H. influenzae*

The kill curve results for moxifloxacin at the MIC are summarized in tables 3.3.2.1 and 3.3.2.2 as well as Fig. 3.3.2.1 – 3.3.2.8. At 10^6 cfu/ml there was growth at every time interval except 6 hours, when there was a 44% reduction in viable cells. Following 6 hours re-growth occurred. The results at 10^7 cfu/ml fluctuated. There was a slight reduction at the 0.5, 1 and 6 hour intervals and growth at the others. At 10^8 cfu/ml there was also sporadic growth and reduction. However, following 24 hours of drug exposure there was a reduction in viable cells by 77%. The 10^9 cfu/ml inoculum began with a substantial reduction of viable cells after 0.5 hours of drug exposure. This was followed by re-growth until 4 hours. The ensuing reduction of viable cells peaked at 99% after 24 hours. The MPC results showed very quick and high levels of reduction for the lower three inocula (Table 3.3.2.4). The 10^6 - 10^8 cfu/ml inocula attained a > 92% reduction of viable cell after 2 hours of drug exposure. The rate of reduction was slower at 10^9 cfu/ml. It was not until 24 hours of drug exposure that a viable cell reduction of 99% was achieved.

The MIC kill results for gatifloxacin are similar to those of moxifloxacin (Table 3.3.2.6, Fig 3.3.2.1 – 3.3.2.8). The 10^6 and 10^7 cfu/ml inocula had a reduction in viable cells of 23 and 16% respectively for the first 2-hours of exposure to the drug. Both of the higher inocula had bacterial reductions of ≥ 46% by 4 hours of drug exposure. By 24 hours, the 10^8 cfu/ml and 10^9 cfu/ml inocula had been reduced by 81% and 98% respectively. At the MPC, the lower three of the four inocula experienced the quickest bacterial reductions (Table 3.3.2.8). At 2 hours of drug exposure, the percentage reduction of viable cells ranged from 85 – 95% for 10^6, 10^7 and 10^8 cfu/ml. By 24 hours the reduction of viable cells reached ≥ 96%. At 10^9 cfu/ml, a reduction of > 93% did not occur until after 12 hours of drug exposure.

With levofloxacin at the MIC drug concentration, re-growth was observed as early as 1 hour after drug exposure for the 10^6 and 10^7 cfu/ml inocula (Table 3.3.2.10, Fig. 3.3.2.1 – 3.3.2.3). This was followed by a slight reduction at 4-6 hours and then more re-growth by 12 hours for the 10^6 cfu/ml inoculum. At 10^8 cfu/ml there was a

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	0.033	0.043	0.098	0.108	-0.43	0.8	1.23
10^7	-0.123	-0.08	-0.0075	-0.33	-0.1	0.24	0.48
10^8	-0.18	0.035	-0.05	0.085	0.063	-0.078	-0.038
10^9	-0.25	-0.32	-0.225	-0.31	-0.66	-1.1	-1.97

Table 3.3.2.1: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to moxifloxacin at the Minimum Inhibition Concentration.
hr. = hour
MIC range of the 4 isolates = 0.004 – 0.031 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	7.49	7.8	37.35	90.06	-44.02	85.44	96.02
10^7	-9.55	-13.08	2.5	7.61	-44.02	38.9	68.3
10^8	-28.12	7.51	-5.67	29.64	26.28	28.95	-77.83
10^9	-40.61	-37.82	-27.35	-41.58	-65.37	-88.87	-98.79

Table 3.3.2.2: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to moxifloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.004 – 0.031 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.53	-1.01	-2.02	-3.95	-4.17	-5.01	-6.47
10^7	-0.5	-1.08	-1.8	-3.3	-3.15	-4.51	-5.32
10^8	-0.22	-0.71	-1.24	-1.4	-1.34	-0.96	-1.6
10^9	-0.09	-0.088	-0.17	-0.2	-0.2	-1.34	-2.35

Table 3.3.2.3: **The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to moxifloxacin at the Mutant Prevention Concentration.**
hr. = hour
MPC range of the 4 isolates = 0.063 – 0.25 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-68.33	-88.44	-97.28	-99.93	-99.99	-100	-100
10^7	-64.65	-88.6	-95.04	-99.87	-99.77	-99.99	-100
10^8	-38.18	-72	-92.37	-94.09	-94.27	-86.78	-95.52
10^9	-15.18	-11.86	-30.49	-34.23	-36.65	-71.42	-99.52

Table 3.3.2.4: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to moxifloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.063 – 0.25 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	0.003	0.045	-0.14	-0.54	-0.65	-0.43	0.53
10^7	0.068	-0.065	-0.15	-0.51	-0.44	-0.72	-0.26
10^8	-0.21	-0.19	-0.24	-0.48	-0.45	-0.5	-0.97
10^9	-0.083	-0.158	-0.31	-0.29	-0.25	-0.8	-1.74

Table 3.3.2.5: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to gatifloxacin at the Minimum Inhibition Concentration.
hr. = hour
MIC range of the 4 isolates = 0.008 – 0.031 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.48	7.99	-23.48	-50.49	-43.12	-66.02	70.11
10^7	13.92	-10.16	-16.26	69	-65.63	-80.9	-37.99
10^8	-22.99	-18.21	-18.88	-52.28	-49.4	-42.02	-80.72
10^9	-14.21	-29.11	-48.02	-46.34	-43.55	-83.1	-98.18

Table 3.3.2.6: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to gatifloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.008 – 0.031 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.33	-0.51	-1.37	-2.28	2.96	-4.19	-5.86
10^7	-0.19	-0.54	-0.98	-1.81	-2.05	-3.1	-1.27
10^8	-0.16	-0.51	-0.92	-1.22	-1.37	-2.2	-1.62
10^9	-0.063	-0.24	-0.19	-0.46	-0.48	-1.17	-2.2

Table 3.3.2.7: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to gatifloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.031 – 0.125 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-48.43	-67.12	-95.31	-99.46	-99.76	-100	-100
10^7	-30.98	-67.37	-86.75	-97.77	-98.95	-99.9	-100
10^8	-29.47	-65.08	-84.6	-89.7	-91.37	-90.62	-96.32
10^9	-7.92	-36.7	-22.26	-65.23	-66.65	-93.19	-99.37

Table 3.3.2.8: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to gatifloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.031 – 0.125 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.015	0.023	0.195	-0.043	-0.115	1.03	1.34
10^7	-0.03	0.03	0.15	0.83	0.6	0.24	0.34
10^8	-0.01	0.058	0.13	0.085	0.13	-0.19	-0.88
10^9	-0.16	-0.17	-0.19	-0.29	-0.43	-1.33	-2.43

Table 3.3.2.9: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to levofloxacin at the Minimum Inhibition Concentration.
hr. = hour
MIC range of the 4 isolates = 0.004 – 0.016 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-3.52	3.79	37.35	-0.52	-22.02	90.1	95.98
10^7	-2.12	7.49	32.79	85.58	75.19	37.79	52.71
10^8	-1.41	13.86	30.22	17.86	30.22	33.8	-79.86
10^9	-14.93	-31.16	-17.86	-42.89	-82.52	-94.4	-99.14

Table 3.3.2.10: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to levofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.004 – 0.016 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.71	-1.03	-1.15	-2.73	-3.51	-4.36	-5.69
10^7	-0.56	-1.01	-1.41	-2.17	-2.45	-3.34	-4.52
10^8	-0.31	-0.97	-1.31	-1.41	-1.36	-1.29	-1.49
10^9	0.24	-0.033	-0.19	-0.19	-0.21	-0.83	-2.07

Table 3.3.2.11: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to levofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.125 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-74.05	-82.56	-93.69	-99.62	-99.96	-99.94	-100
10^7	-68.12	-83.53	-90.53	-98.89	-99.25	-99.91	-100
10^8	-37.79	-80.83	-88.87	-91.48	-90.83	-88.63	-95.86
10^9	38.2	13.69	-33.97	-35.05	-39	-83.15	-94.35

Table 3.3.2.12: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to levofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.125 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	0.01	0.067	0.25	0.59	no data	2.46	1.54
10^7	-0.05	-0.055	0.12	1.26	0.52	0.5	0.38
10^8	-0.013	-0.083	-0.067	0.05	0.1	-0.065	-0.96
10^9	0.067	0.013	-0.02	-0.057	-0.093	-0.8	-2

Table 3.3.2.13: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to ofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.008 – 0.0.16 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	3.07	13.88	44.15	71	no data	99.66	97.03
10^7	-8.05	-11.16	32.1	93.41	69.77	69	52.23
10^8	-2.14	-15.38	-12.19	10.1	29.67	-9.7	-87.45
10^9	13.88	-2.14	-4.2	-11.66	-26.73	-76.66	-99.5

Table 3.3.2.14: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to ofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.008 – 0.016 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.32	-0.84	-1.73	-2.74	-2.99	-3.9	-4.55
10^7	-0.25	-0.58	-1.44	-2.25	-2.55	-2.92	-3.94
10^8	-0.19	-0.62	-0.95	-0.91	-0.86	-0.64	-1.27
10^9	-0.015	0.01	-0.065	-0.14	-0.21	-1.11	-2.23

Table 3.3.2.15: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to ofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.125 – 0.25 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-46.24	-76.89	-93.96	-99.39	-99.66	-99.92	-99.95
10^7	-28.76	-68.01	-89.2	-98.19	-99.1	-99.77	-99.96
10^8	-30.61	-69.47	-83.7	-85.14	-82.78	-75.44	-93.77
10^9	9.84	10.09	-5.92	-12.06	-32.73	-91.04	-99.27

Table 3.3.2.16: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to ofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.125 – 0.25 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	0.033	0.03	0.21	0.42	0.79	1.58	1.52
10^7	-0.028	0.065	0.21	0.47	0.75	0.82	0.56
10^8	-0.0025	0.065	0.205	0.288	0.255	0.158	-0.7
10^9	0.035	0.068	0.088	-0.018	-0.085	-0.78	-2.84

Table 3.3.2.17: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to ciprofloxacin at the Minimum Inhibition Concentration.
hr. = hour
MIC range of the 4 isolates = 0.002 – 0.008 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	7.49	7.49	37.35	57.9	82.9	97.24	97
10^7	-3.95	13.88	37.35	60.37	81.3	85.64	71.63
10^8	1.44	13.88	37.34	38.3	37.9	97.03	-74.35
10^9	7.6	13.91	17.14	-2.9	-17.29	-83.34	-98.52

Table 3.3.2.18: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to ciprofloxacin at the Minimum Inhibitory Concentration.
hr. = hour
MIC range of the 4 isolates = 0.002 – 0.008 μg/ml

	Ave. Log Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-0.38	-0.77	-1.4	-2.16	-2.4	-3.25	-4.67
10^7	-0.21	-0.77	-1.1	-1.81	-2.14	-3	-4.72
10^8	-0.22	-0.48	-0.62	-0.165	-0.23	-0.74	-1.01
10^9	0.073	-0.113	-0.16	-0.165	-0.23	-0.9	-2.01

Table 3.3.2.19: The average $\log_{10}$ reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to ciprofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.063 – 0.125 μg/ml

	Ave. Percentage Reduction						
Inoculum (cfu/ml)	**0.5 hr.**	**1 hr.**	**2 hr.**	**4 hr.**	**6 hr.**	**12 hr.**	**24 hr.**
10^6	-55.01	-71.29	-88.81	-97.06	-98.05	-99.71	-100
10^7	-36.4	-62.05	-84.16	-93.93	-96.92	-99.41	-100
10^8	-35.3	-53.48	-68.81	-69.84	-71.29	-71.7	-88.38
10^9	37.62	-16.24	-29.57	-29.99	-40.84	-85.49	-98.93

Table 3.3.2.20: The average percentage reduction in viable cells of 4 clinical *H. influenzae* isolates exposed to ciprofloxacin at the Mutant Prevention Concentration.
hr. = hour
MPC range of the 4 isolates = 0.063 – 0.125 μg/ml

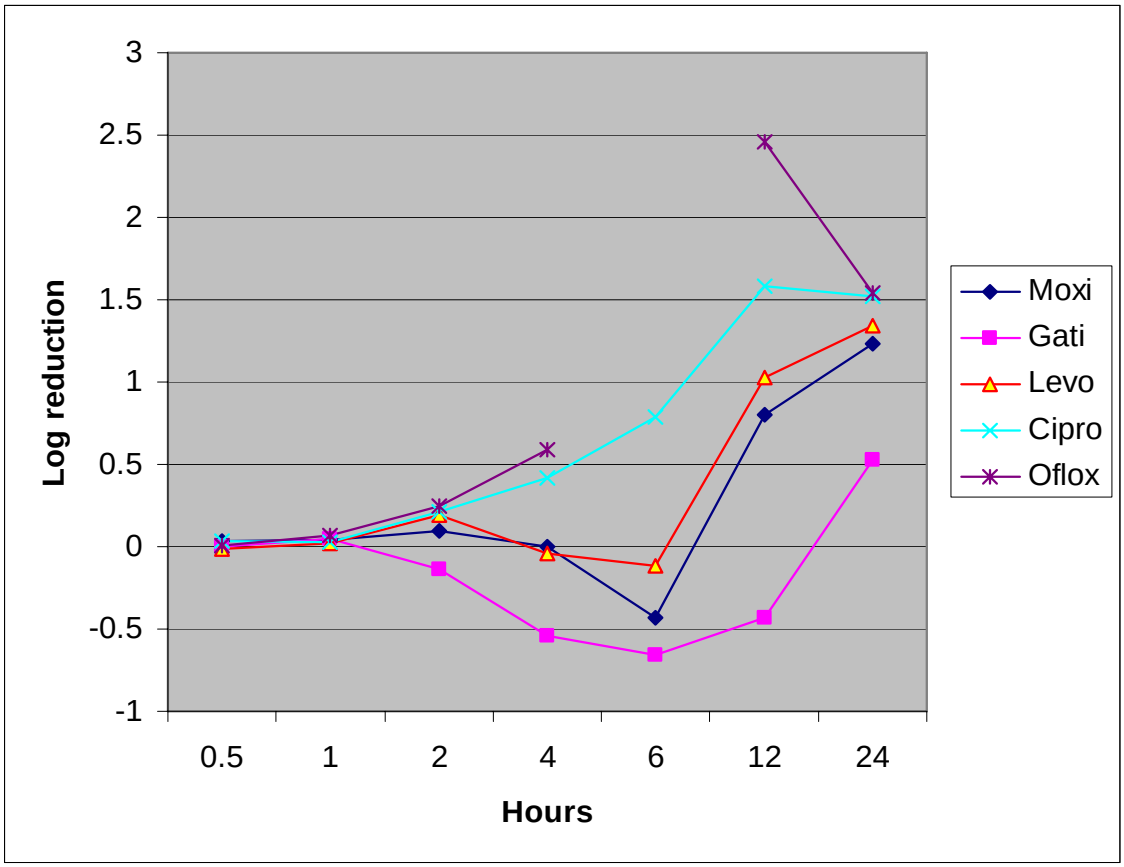

Figure 3.3.2.1: The Killing of *H. influenzae* ocular isolates (n = 4) at 10^6 cfu/ml following exposure to each of the five fluoroquinolones at the Minimum Inhibitory drug concentration. (data was not determined for ofloxacin at 6 hrs)

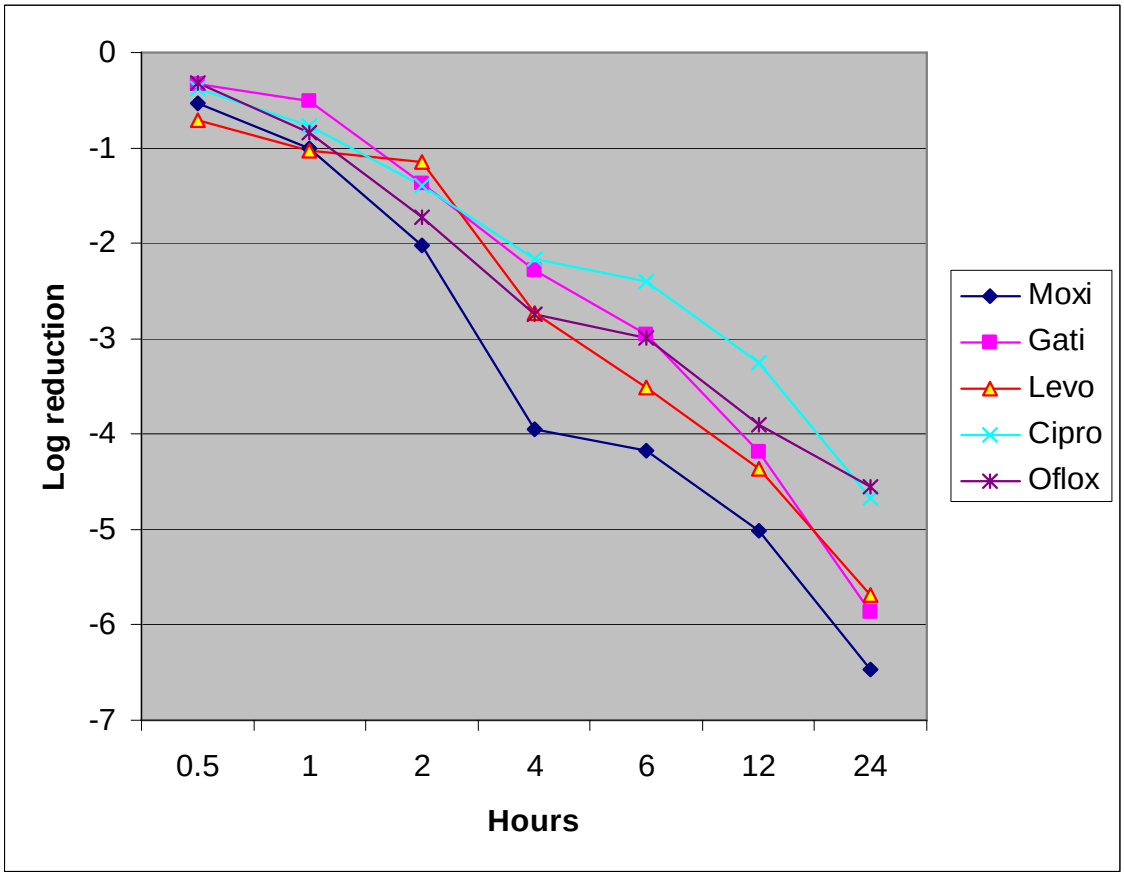

Figure 3.3.2.2: The Killing of *H. influenzae* ocular isolates (n = 4) at 10^6 cfu/ml following exposure to each of the five fluoroquinolones at the Mutant Prevention drug concentration.

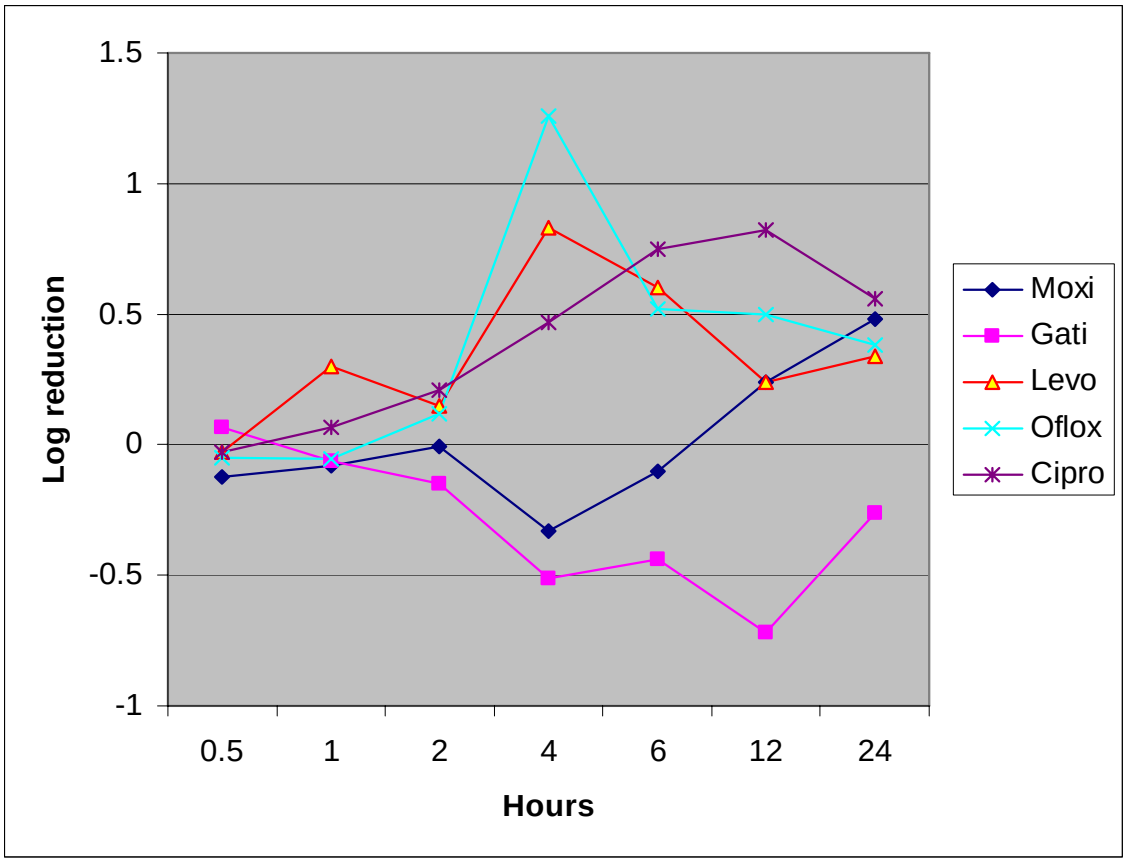

Figure 3.3.2.3: The Killing of *H. influenzae* ocular isolates (n = 4) at 10^7 cfu/ml following exposure to each of the five fluoroquinolones at the Minimum Inhibitory drug concentration.

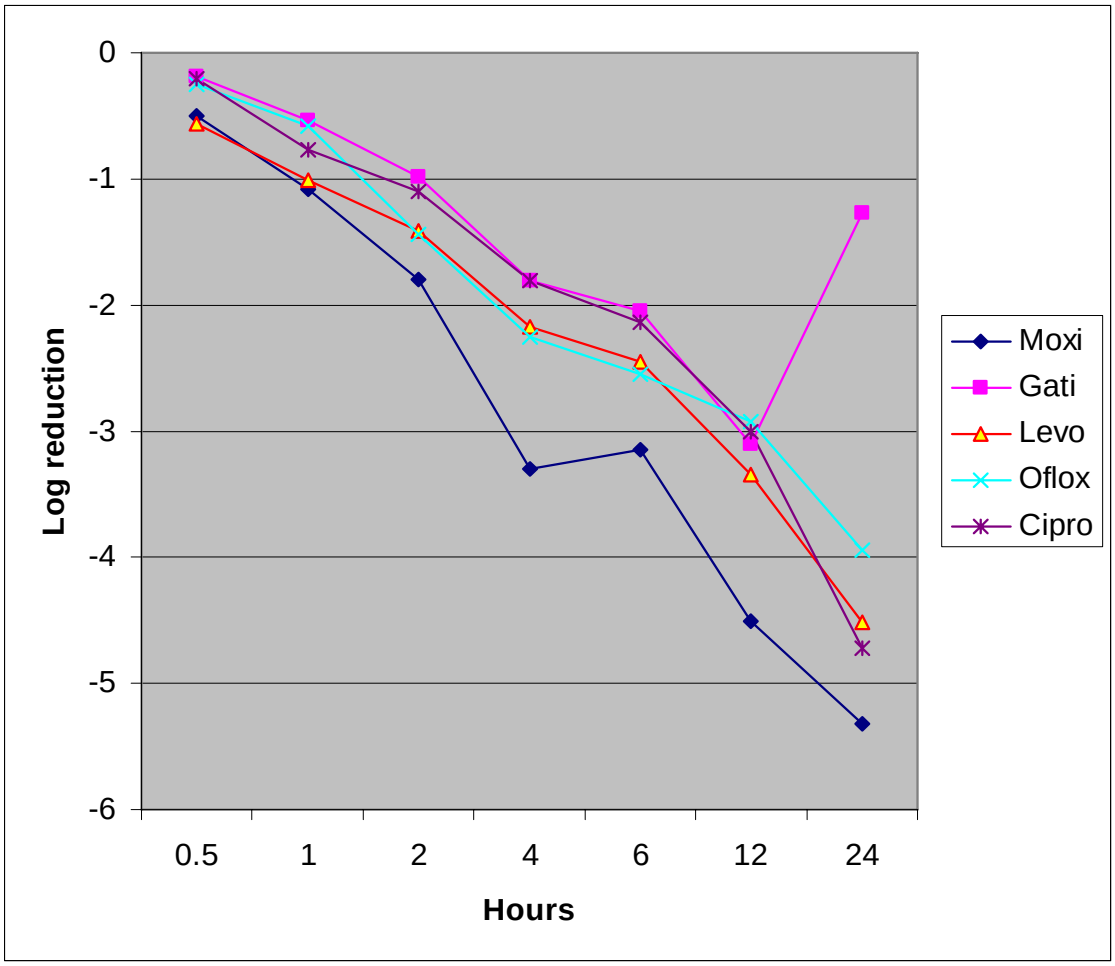

Figure 3.3.2.4: The Killing of *H. influenzae* ocular isolates (n = 4) at 10^7 cfu/ml following exposure to each of the five fluoroquinolones at the Mutant Prevention concentration.

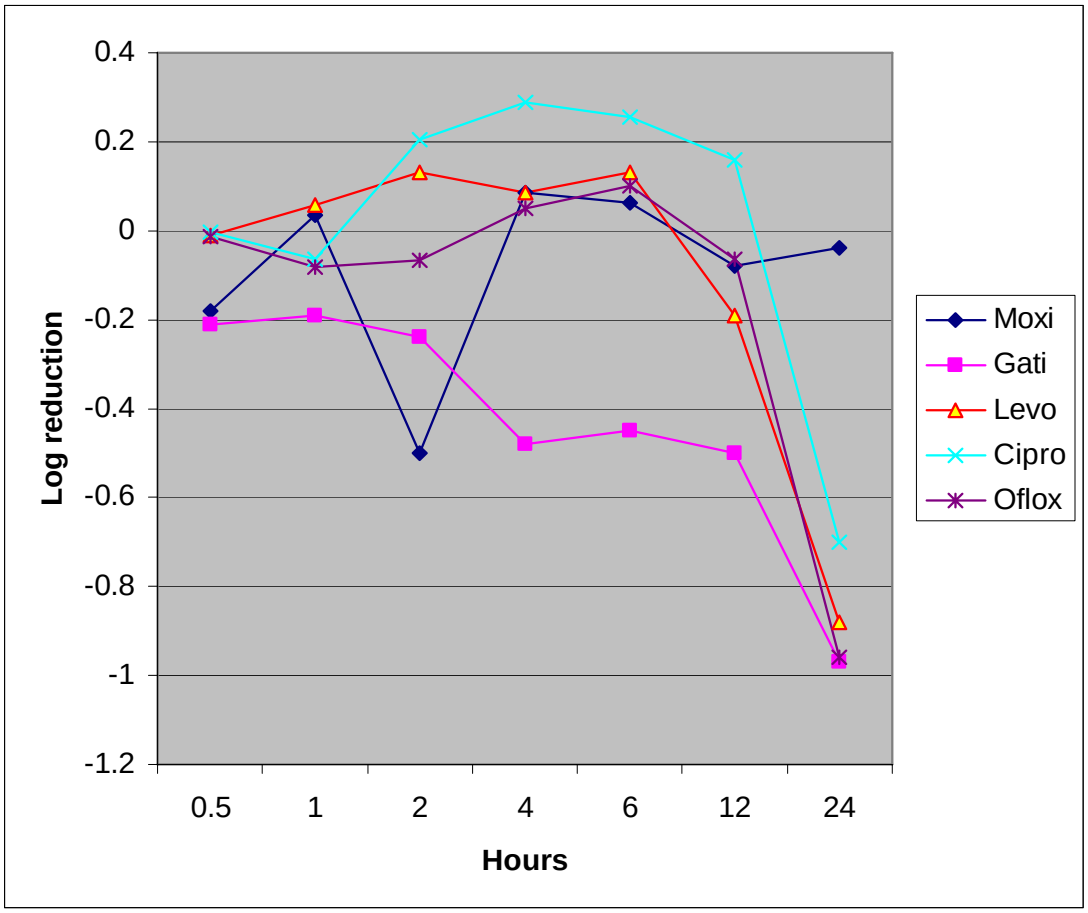

Figure 3.3.2.5: The Killing of *H. influenzae* ocular isolates (n = 4) at 10^8 cfu/ml following exposure to each of the five fluoroquinolones at the Minimum Inhibitory drug concentration.

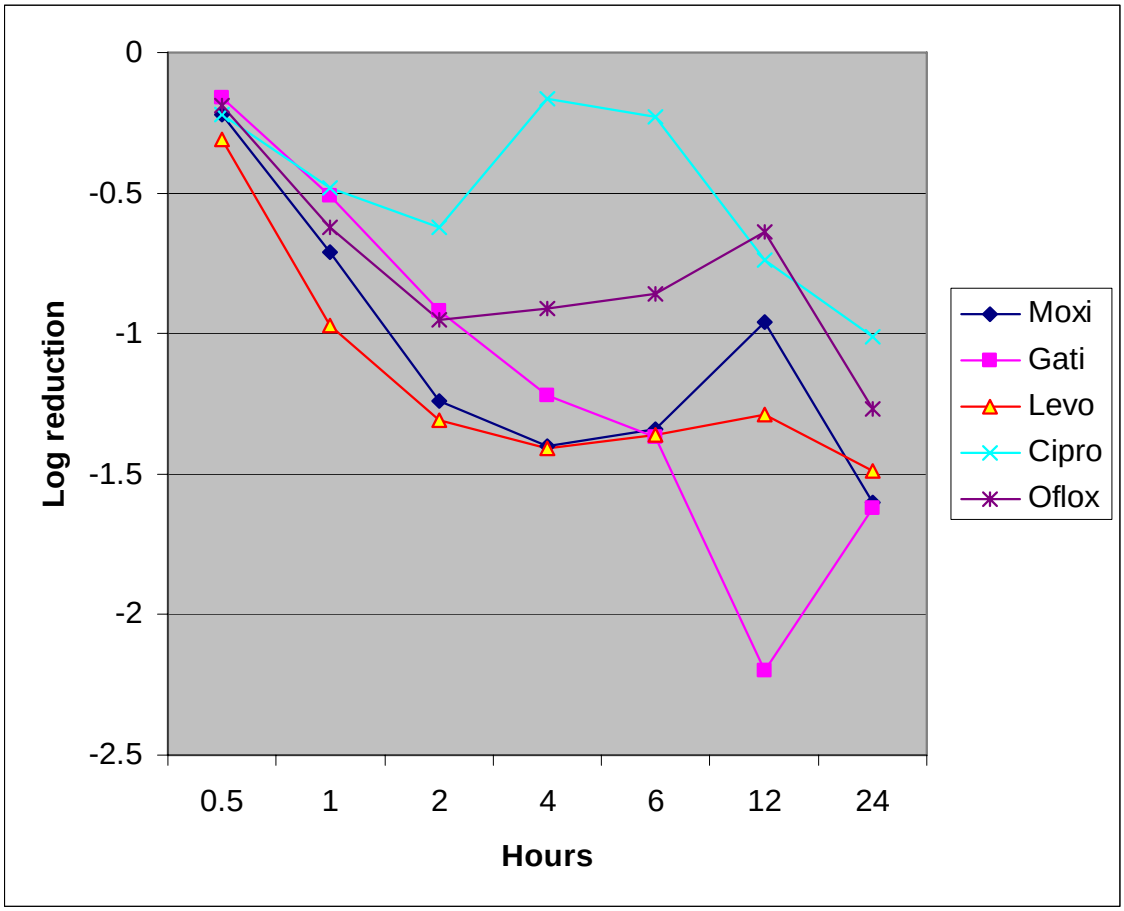

Figure 3.3.2.6: The Killing of *H. influenzae* ocular isolates (n = 4) at 10^8 cfu/ml following exposure to each of the five fluoroquinolones at the Mutant Prevention drug concentration.

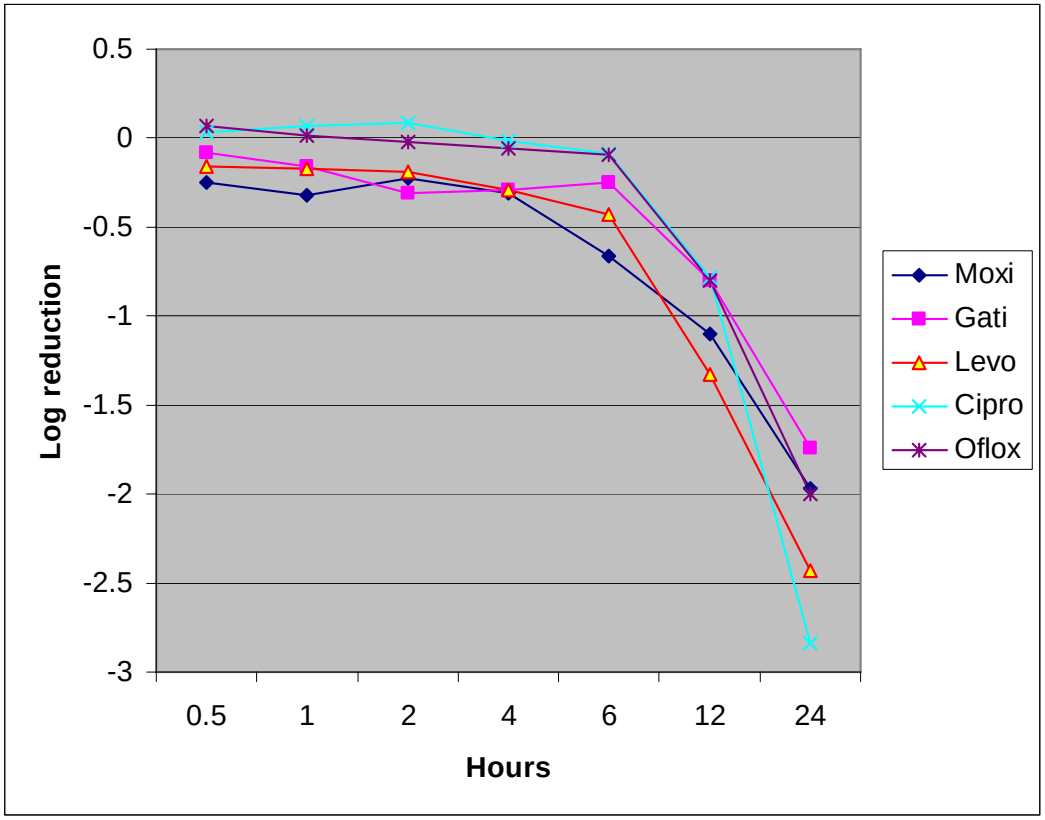

Figure 3.3.2.7: The Killing of *H. influenzae* ocular isolates (n = 4) at 10^9 cfu/ml following exposure to each of the five fluoroquinolones at the Minimum Inhibitory drug concentration.

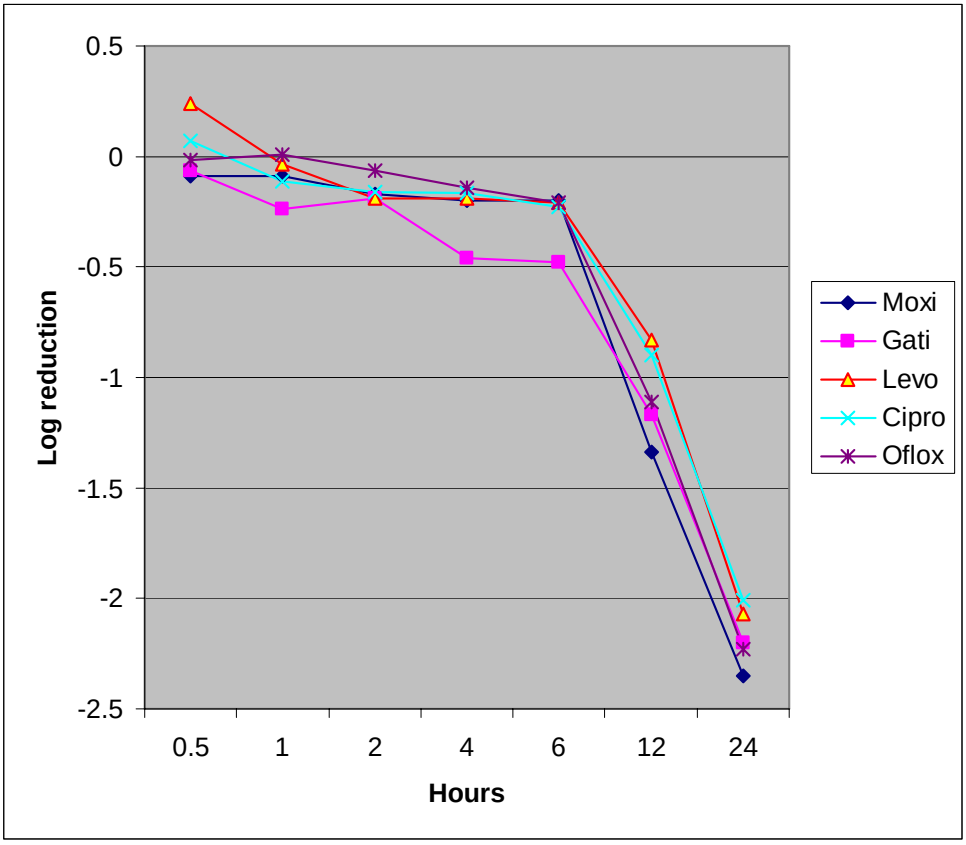

Figure 3.3.2.8: The Killing of *H. influenzae* ocular isolates (n = 4) at 10^9 cfu/ml following exposure to each of the five fluoroquinolones at the Mutant Prevention drug concentration.

reduction of viable cells which amounted to 80% following 24 hours of drug exposure. The largest inoculum, however, was reduced by 83% at 6 hours and 99% by 24 hours. At the MPC concentration, a significant reduction occurred early on for the $10^6 - 10^8$ cfu/ml inocula (Table 3.3.2.12, Fig. 3.3.2.1 – 3.3.2.6). As early as 1 hour after drug exposure, there was a reduction in viable cells of ≥ 80% for these lower inocula. The equivalent reduction at 10^9 cfu/ml was not observed until after 12 hours of drug exposure.

Despite exposure to ofloxacin at the MIC drug concentration, significant growth was seen throughout the 24 hours of drug exposure at 10^6 cfu/ml (Table 3.3.2.14, Fig. 3.3.2.1). The 10^7 cfu/ml inoculum began with a slight reduction for the first two sampling intervals but began to re-grow after 2 hours. There was no substantial reduction in viable cells for 10^8 cfu/ml until 24 hours of drug exposure when a reduction of 87% was noted. At 10^9 cfu/ml, there was noticeable reduction in viable cells by 77%, which began at 12 hours following drug exposure and reached 99.5% by 24 hours. Following exposure to ofloxacin at the MPC, significant bacterial killing in the $10^6 - 10^8$ cfu/ml inocula was evident after 2 hours of drug exposure. By 12 hours the 10^9 cfu/ml inoculum had sustained a reduction in viable cells by 91% and by 24 hours, all inocula had sustained a viable cell reduction of ≥ 93%.

As with the previous four fluoroquinolones at the MIC drug concentrations, the $10^6 - 10^7$ cfu/ml inocula proliferated throughout the 24 hours of exposure to ciprofloxacin (Table 3.3.2.18, Fig. 3.3.2.1 – 3.3.2.3). No reduction in viable cells was observed at 10^8 cfu/ml until 24 hours of drug exposure. The reduction of viable cells at 10^9 cfu/ml began at 4 hours with a reduction of 3% and increased to 99% by 24 hours. The 10^6 and 10^7 cfu/ml inocula had a reduction of viable cells by ≥ 84% following 2 hours at the MPC drug concentration (Table 3.3.2.20). By 24 hours of drug exposure the $10^6 - 10^7$ cfu/ml inocula had been reduced by 100%. With the 10^8 and 10^9 cfu/ml inocula, bacterial reduction was 88% and 98% respectively by 24 hours of drug exposure.

4.0 Discussion

Sight is considered to be one of the most significant senses that humans possess. As a result, anything that threatens to compromise our visual integrity needs to be taken very seriously. Bacterial infections pose such a threat. While both Gram-positive and Gram-negative organisms are responsible for causing infections like conjunctivitis, keratitis and endophthalmitis, certain bacteria are more frequently recovered from ocular specimens than others. In cases of conjunctivitis as many as 92% of recovered bacterial isolates are Gram-positive organisms such as *S. pneumoniae* and *S. aureus* (4). In pediatric conjunctivitis cases, *H. influenzae* and *S. pneumoniae* are most common with prevalence rates of 73% and 20% respectively (83). With keratitis, Gram-positive bacteria are also the most prevalent pathogens with organisms like *Staphylococcus epidermidis*, *S. aureus* and *S. pneumoniae* being recovered 40%, 22% and 8% of the time respectively (29). In endophthalmitis infections, the most prevalent pathogens are Gram-negative at 41.7%, with the most common one being *P. aeruginosa*. For endophthalmitis Gram-positives are present 37.6% of the time with *S. epidermidis* being the most likely organism of this group (84).

Despite the availability of several antimicrobial classes for treatment of ocular infections, fluoroquinolones, due to their broad-spectrum activity, are especially effective (33). However, like with any antimicrobial drugs, clinical use has resulted in the development of resistance (44). This is exactly what has been observed with the older fluoroquinolones ciprofloxacin, ofloxacin and levofloxacin. For example, Goldstein *et al* (85) showed that in *S. aureus* isolates from bacterial keratitis specimens resistance to ciprofloxacin and ofloxacin increased 6 to 7-fold between 1993 and 1997. Similar trends have been noted with *S. pneumoniae*. A study done in the United States during 2000 assessed the susceptibility of 4009 *S. pneumoniae* isolates recovered from blood, respiratory, eye, ear and body fluid specimen. Overall they found that only 71% of the isolates were sensitive to ciprofloxacin (86).

In response, drug companies have worked to develop newer fluoroquinolones with improved *in vitro* Gram-positive activity. This has resulted in two new agents being introduced, moxifloxacin licensed by Alcon, and gatifloxacin, currently licensed by Allergan. Both drugs have a methoxy side-chain added at the R_8 position. In

addition, moxifloxacin has a bulky bicyclic ring attached at the R_7 position while gatifloxacin has a methyl group on its piperazinyl ring (51). These changes result in increased Gram-positive activity (lower MICs) as well as creating dual target activity. While the older fluoroquinolones interfere with either DNA gyrase or topoisomerase IV, moxifloxacin and gatifloxacin interfere with both simultaneously (51). This means that these new agents can potentially eradicate organisms that possess a mutation in the genes that encode for one of the two of the fluoroquinolone targets. In other words, an infection caused by an organism that has become resistant to older fluoroquinolones can, theoretically, be treated with either gatifloxacin or moxifloxacin. The newer fluoroquinolones have also been equipped with bulky side-chains making them less susceptible to bacterial efflux mechanisms such as NorA efflux membrane protein.

In the first part of my project, I attempted to compare the *in vitro* potency of fluoroquinolones' towards bacteria pertinent to ocular infections. Two of the most common Gram-positive organisms, *S. pneumoniae* and *S. aureus*, and two of the most common Gram-negative organisms, *H. influenzae* and *P. aeruginosa*, associated with ocular infections were used. While the vast majority of susceptibility testing is done using the MIC method, it was thought that it was necessary to do MPC testing as well. While MIC testing is rapid and convenient (test results are available in 24 hours) it does not determine the susceptibility of a bacterial population that is most often present in an *in vivo* infection. MIC tests employ a standardized test inoculum of 10^5 cfu/ml. However, this underestimates the true size of an *in vivo* bacterial load which can reach sizes of 10^{10} cfu in infections (81). What is the significance of this underestimation? With the organisms used in this project, the spontaneous mutation frequency is approximately 10^{-7} to 10^{-9}. Therefore, if a person is infected with a bacterial load of 10^9 cfu, that population is likely to have at least one organism with a first-step resistance mutation. Because current dosing strategies have been determined using MIC susceptibility results, the prescribed dosage for an infection is based on a standard population of bacteria of 10^5 cfu in size; a population not large enough for detecting first-step mutations that may arise. Therefore the dosage approved may not be taking into consideration the presence of organisms with mutations. As a result the dosage may be sufficient to kill the susceptible organisms within the population but may not be

high enough to kill the resistant ones, leading to selective enrichment of this resistant sub-population of bacteria. This may be one mechanism by which global resistance is occurring to fluoroquinolones and perhaps other compounds as well.

While MPC testing may prove more accurate in assessing the true susceptibility of an organism, due to the large inoculum (10^9 cfu), there are some aspects to the test which keep it from being a realistic replacement for MIC testing. The main hindrances lie in the conduction of the test itself. MPC testing takes five to six days to complete and is more expensive and technically demanding.

Several observations were made based on the results of the susceptibility testing done in this project. First, both the MIC and MPC results confirmed that, indeed, the new fluoroquinolones have better *in vitro* Gram-positive activity (lower MICs) against ocular pathogens. Gatifloxacin and moxifloxacin were approximately four-times more active than the older fluoroquinolones for both *S. pneumoniae* and *S. aureus*. Secondly, trends shown by the MIC results were reflected in the MPC results. For instance, the increase in Gram-positive activity of the new fluoroquinolones against *S. pneumoniae* was between 4 and 8-fold as shown by the MIC results. While the actual values were different for the MPC tests, the new fluoroquinolone values were shown to have the same-fold increase in Gram-positive activity (lower MPCs) as displayed by the MIC test. Experiments done by Kowalski *et al* (47). show virtually identical MIC results for ocular *S. pneumoniae* isolates. Their data showed MIC_{90} values for moxifloxacin and gatifloxacin at 0.19 and 0.25 µg/ml respectively, while the older fluoroquinolones had MIC_{90} values which were between 4 and 8-fold higher at 2 and 4 µg/ml for ofloxacin and ciprofloxacin respectively. In 2001 Blondeau *et al*. (81) published MPC data on clinical *S. pneumoniae* isolates. While their actual MPC_{90} values were slightly different than those determined in this thesis project, the increased *in vitro* potency of the new fluoroquinolones was observed. Their data showed a 2 to 4-fold increase in the *in vitro* potency of gatifloxacin and moxifloxacin over levofloxacin.

The Gram-negative results were expected to be different than the Gram-positive results. As the new fluoroquinolones were designed to be more active against Gram-positives, I was not expecting to see a difference in *in vitro* Gram-negative activity. This is precisely what was observed. As a whole, the MIC results for *P. aeruginosa*

were higher than for the other three organisms. This is not a good sign considering *P. aeruginosa* is the leading cause of keratitis in contact lens associated keratitis (33). The reason the *Pseudomonas* results seemed high, however, is because the population of organisms I tested contained three fluoroquinolone-resistant isolates based on recently published fluoroquinolone breakpoints presented by Morrissey *et al.* (87). Because ciprofloxacin is the only fluoroquinolone, of the five tested, to be considered anti-pseudomonal, the newer fluoroquinolones were expected to have less *in vitro* potency. Despite there being no increase in *in vitro* activity for the new fluoroquinolones, with respect to *H. influenzae*, the five fluoroquinolones were still very active against this organism.

The results of the MPC testing from this project helped support the hypothesis that MIC results may be underestimating the actual drug concentration needed to eliminate susceptible and first-step resistant populations of bacteria which may be present in an *in vivo* infection. The MPC results were consistently two to four-fold higher than the MIC results. One additional point that needs to be emphasized is that current antimicrobial susceptibilities are based on PK/PD information from serum analysis (80). However, in the case of superficial ocular infections like conjunctivitis and keratitis, drug concentrations greatly in excess of systemic limitations are achievable at the site of infection via topical application. As a result, an organism that is considered resistant via traditional MIC testing, may be treatable if present as a superficial ocular pathogen.

While the first two experimental approaches examined the ability of five fluoroquinolones to inhibit the growth of typical ocular pathogens (MIC), and, inhibit the growth of first-step resistant mutants (MPC), the kill curve component was used to determine whether MIC or MPC drug concentrations were suitable for killing, *S. pneumoniae* and *H. influenzae* ocular isolates. Previous kill studies done by Blondeau *et al* (88) showed a more rapid reduction in viable cells at the MPC than the MIC drug concentrations. This same observation was made during my experiments. With *S. pneumoniae* there was a rather dramatic difference both in the amount of time it took for a significant reduction of viable cells (99.9%) and the length of time the reduction lasted. For example, at the MIC there was rarely a reduction of 99.9% of viable cells

before 12 hours, if at all. In the cases where there was a significant reduction, it was followed by re-growth by 24 hours of drug exposure. At the MPC drug concentration, there was a reduction by at least 99% for every drug at every inoculum size. This effect was observable most often by 6 hours of drug exposure, and sometimes as early as 4 hours of drug exposure with gatifloxacin and moxifloxacin. At no time was any re-growth observed at the MPC. Ibrahim *et al*. (89) also conducted kill studies with *S. pneumoniae*. While they did not utilize the MPC concept, they did conduct kill curves using concentrations at and above the MIC. They too found that re-growth was a characteristic found only at the MIC drug concentration. With drug concentrations in excess of the MIC, re-growth was not observed.

With *H. influenzae* there was even less killing at the MIC drug concentration than observed with *S. pneumoniae* clinical isolates. There were only two instances where a 99% reduction of viable cells occurred. The first was with levofloxacin at 10^9 cfu/ml, and the second was with ofloxacin at 10^9 cfu/ml. In both cases the 99% reduction did not occur until 24 hours following drug exposure. Re-growth was evident consistently in the $10^6 – 10^7$ cfu/ml inocula for each of the five fluoroquinolones. While the rate of killing was faster at the MPC drug concentration, significant reductions were primarily observed in the lower two inocula and not until 6 – 12 hours. Although significant reductions were not observed for the higher inocula, no re-growth occurred. Had there been a longer test period, the larger inocula may have reached a 99% reduction in viable cells following longer intervals of drug exposure. Unfortunately, because the experimental method utilized to conduct these kill curves was relatively new I was unable, without further investigation, to explain why multiple growth fluctuations were observed throughout a couple of the kill tests.

Overall, the results from this project have shown that gatifloxacin and moxifloxacin do have significantly increased *in vitro* Gram-positive activity compared to the older compounds. While *in vitro* Gram-negative activity seems to be well preserved amongst all the five fluoroquinolones tested, the results showed that perhaps *P. aeruginosa*, remains a problematic organism for the fluoroquinolone class as a whole. However, to make such a generalization, one would need to conduct a multi-

center study with hundreds of *P. aeruginosa* isolates using the most commonly prescribed fluoroquinolones and compare *in vitro* results to clinical outcome.

It was also shown that the MPC test more accurately represents the drug concentration needed to eradicate the type bacterial populations present in an *in vivo* infection. This means that the MPC test takes into consideration the actual size of the bacterial load which may be present in the average infection. As well, it takes into account the presence of first-step mutants which may be present in any bacterial inoculum which is greater than 10^6 cfu.

5.0 Future Considerations

Unfortunately, due to the time restraints of this project, it was not possible to examine all the aspects of drug performance within an ocular setting. While I was able to compare the *in vitro* potency of gatifloxacin and moxifloxacin to older fluoroquinolones like ciprofloxacin, ofloxacin and levofloxacin, there are additional questions that need to be investigated. For example, an animal model could be used in order to determine the ability of the new fluoroquinolones to maintain therapeutic concentrations in the tear film. As well, the five fluoroquinolones need to be examined for their ability to penetrate the cornea following standardized topical administration. If sufficient corneal penetration is achieved, we need to see how long it takes to reach therapeutic concentrations in the aqueous and vitreous humors.

With regards to the kill curve experiments, further investigation needs to be done to find out what is actually going on in each test tube. High Performance Liquid Chromatography (HPLC) needs to be used to determine how quickly the nutrients in the broth as well as the drugs, are being used up over the 24 hour test period. With this instrument you can measure the amount of drug or nutrient in the sample. This is important because in order to test the true pharmacodynamics, there has to be sufficient nutrient and drug concentrations present throughout the entire test period.

6.0 REFERENCES

1. **A Sheikh, B Hurwitz.** Topical antibiotics for acute bacterial conjunctivitis: a systemic review. *British Journal of General Practice*. 2001;51:473-7.
2. **J D Bartlett, SD Jaanus.** *Clinical Ocular Pharmacology*. 4 ed Woburn MA: Butterworth Heinman; 2001.
3. **DV Seal, JI McGill, IA Maclie, GM Liakos, P Jacobs, NJ Goulding.** Bacteriology and tear protein profiles of the dry eye. *British Journal of Ophthalmology*. 1986;70(2):122-5.
4. **SL Everett, RP Kowalski, LM Karenchak, D Landsittel, R Day, YJ Gordon.** An *in vitro* comparison of the susceptibilities of bacterial isolates from patients with conjunctivitis and blepharitis to newer established topical antibiotics. *Cornea*. 1995;14(4):382-7.
5. **C Miller, PL Kaufman.** Aqueous humor: secretion dynamics. In: EA Jaeger, ed. *Duane's Foundation of Clinical Ophthalmology*. Philadelphia: Lippincott-Raven; 1995.
6. **D Lamberts.** Physiology of the tear film. In: G. Smolin RT, ed. *The Cornea*. Boston: Brown; 1994.
7. **N Nicolaides.** Recent findings on the chemical composition of the lipids of steer and human meibomian glands. In: holly F, ed. *The preocular tear film in health, disease, and contact lens wear*. Lubbock: Dry Eye Institute; 1986.
8. **A Mircheff.** Water and electrolyte secretion and fluid modification. In: D.M. Albert FJ, N. Robinson, ed. *Ophthalmology: basic sciences*. Philadelphia: WB Saunders; 1994.
9. **PL Kaufman, A ALM.** *Adler's physiology of the eye. Clinical application.* 10th ed St. Louis: Mosby; 2003.
10. **C Hanna, DS Bicknell, J O'Brien.** Cell turnover in the adult human eye. *Archives of Ophthalmology*. 1961;65:695.
11. **JL Funderburgh, ML Funderburgh, SJ Brown, JP Vergnes, JR Hassell, MM Mann, GW Conrad.** Sequence and structural implications of a bovine keratin sulfate proteoglycan core protein: protein 37B represents bovine lumican and proteins 37A and 25 are unique. *Journal of Biological Chemistry*. 1993;268:11874.
12. **D Sevel.** A reappraisal of the development of the eyelids. *Eye*. 1988;2:123.
13. **EA Boettner, JR Wolter.** Transmission of the ocular media. *Investigative Ophthalmology & Visual Science*. 1962;6:776.
14. **D Gospodarowicz, G Greenburg, I Vlodavsky, J Alvarado, LK Johnson.** Identification and location of fibronectin in cultured corneal epithelial cells: cell surface polarity and physiological implications. *Experimental Eye Research*. 1979;29:485.
15. **JJ Kanski.** *Clinical ophthalmology a systemic approach*. 5th ed Butterworth - Heinmann; 2003.
16. **BJ McLaughlin, MD McCartney, TO Wood.** Freeze fracture quantitative comparison of rabbit corneal epithelial and endothelial membranes. *Current Eye Research*. 1985;4:951.
17. **A Bill.** Basic physiology of the drainage of aqueous humor. In: LZ Bito HD, ed. *The ocular and cerebrospinal fluids: Fogarty International Center Symposium*. London: Academic Press; 1977.
18. **A Bill.** The role of ciliary blood flow and ultrafiltration in aqueous humor formation. *Experimental Eye Research*. 1973;16:287.
19. **H Davson.** The aqueous humor and the intraocular pressure. In: H Davson, ed. *Physiology of the eye*. New York: Pergamon Press; 1990.
20. **JP Leeming.** Treatment of ocular infections with topical antibacterials. *Clinical Pharmacokinetics*. 1999;37:351-60.
21. **EA Balazs, JL Denlinger.** The vitreous. In: H Davson, ed. *The Eye*. Vol. 1A. New York: Academic Press; 1972.
22. **I Fatt.** Flow and diffusion in the vitreous body of the eye. *Bulletin of Mathematical Biology*. 1975;37:85.
23. **M Bradbury, L Lightman.** The blood brain interface. *Eye*. 1990;4:249.
24. **TR Singer, SJ Isenberg, L Apt.** Conjunctival anaerobic and aerobic bacterial flora in pediatric versus adult subjects. *British Journal of Ophthalmology*. 1988;72(6):448-51.
25. **DF Larkin, JP Leeming.** Quantitative alterations of the commensal eye bacteria in contact lens wear. *Eye*. 1991;5:70-4.
26. **M Yanoff, BS Fine.** *Ocular pathology* Philadelphia: JB Lippencott; 1989.

27. **MH Friedlaender.** *Allergy and immunology of the eye* New York: Raven Press; 1993.
28. **JL Baum.** Antibiotic use in ophthalmology. In: EA Jaeger, ed. *Duane's clinical ophthalmology*. Philadelphia: JB Lippincott; 1993.
29. **F Schaefer, O Bruttin, L Zografos, Guex-Crosier Y.** Bacterial keratitis: a prospective clinical and microbiological study. *British Journal of Ophthalmology*. 2001;85:842-7.
30. **MB Limberg.** Diseases of the cornea. In: CW Mitchell, ed. *Diseases of the external eye and adnexa*. Baltimore: Williams & Wilkins; 1993.
31. **GM Bohigian.** *Handbook of external diseases of the eye*. 3rd ed Thorofare, N.J.: Slack; 1987.
32. **TD Matthews, DD Fraser, DC Minassian, CF Radford, JK Dart.** Risks of keratitis and patterns of use with disposable contact lenses. *Archives of Ophthalmology*. 1992;110:1559-62.
33. **RA Armstrong.** The microbiology of the eye. *Ophthalmic and Physiological Optics*. 2000;20:421-41.
34. **LJ Catania.** Diagnosis of the cornea. In: Norwalk C, ed. *Primary care of the anterior segment*. 2nd ed: Appleton & Lange; 1995.
35. **L Bonomi, S Perfetti, R Bellucci, F Massa, I DeFranco.** Prevention of surgically induced miosis by diclofenac eye drops. *Annals of Ophthalmology*. 1987;19:142-5.
36. **SC Zantos.** Management of corneal infiltrates in extended-wear contact lens patients. *Int. Contact Lens Clin*. 1984;10:604-10.
37. **PA Bloom, JP Leeming, W Power, DH Laidlaw, LT Collum, DL Easty.** Topical ciprofloxacin in the treatment of blepharitis and blepharoconjunctivitis. *European Journal of Ophthalmology*. 1994;4(1):6-12.
38. **DM Maurice.** The dynamics and drainage of tears. *International Ophthalmology Clinics*. 1973;3:103-16.
39. **SS Chrai, MC Makoid, SP Eriksen, JR Robinson.** Drop size and initial dosing frequency problems of topically applied ophthalmic drugs. *Journal of Pharmaceutical Sciences*. 1974;63:333-8.
40. **SD Holmberg, SL Solomon, PA Blake.** Health and economic impacts of antimicrobial resistance. *Reviews of Infectious Diseases*. 1987;9:1065 - 78.
41. **WM Scheld.** Maintaining fluoroquinolone class efficacy: review of influencing factors. *Emerging Infectious Diseases*. 2003;9:1 - 9.
42. **CG Whitney, MM Farley, J Harder.** Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States. *New England Journal of Medicine*. 2000;343:214 - 19.
43. **DC Hooper.** Fluoroquinolone resistance among Gram-positive cocci. *THE LANCET Infectious Diseases*. 2002;2.
44. **G Alexandrakis, EC Alfonso, D Miller.** Shifting trends in bacterial keratitis in South Florida and emerging resistance to fluoroquinolones. *Ophthalmology*. 2000;107:1497 - 502.
45. **FB Marangon, D Miller, Muallem M, AC Romano, EC Alfonso.** Ciprofloxacin and levofloxacin resistance among methicillin-sensitive *Staphylococcus aureus* isolates from keratitis and conjunctivitis. *American Journal of Ophthalmology*. 2003;137(3):453-8.
46. **MR Chalita, AL Hofling-Lima, *et al*.** Shifting trends in *in vitro* antibiotic susceptibilities for common ocular isolates during a period of 15 years. *American Journal of Ophthalmology*. 2004;137(1):43-51.
47. **RP Kowalski, DK Dhaliwal, LA Karenchak, EG Romanowski, FS Mah, DC Ritterband, J Gordon.** Gatifloxacin and moxifloxacin: an in vitro susceptibility comparison to levofloxacin, ciprofloxacin and ofloxacin using bacterial keratitis isolates. *American Journal of Ophthalmology*. 2003;136:500 - 5.
48. **A Graves, M Henry, T O'Brien, DG Hwang, A Van Buskirk, MD Trousdale.** In vitro susceptibilities of bacterial ocular isolates to fluoroquinolones. *Cornea*. 2001;20:301 - 05.
49. **P Ball.** Quinolone generations: natural history or natural selection. *Journal of Antimicrobial Chemotherapy*. 2000;46:17-24.
50. **CM Oliphant, GM Green.** Quinolones: a comprehensive review. *American Family Physician*. 2002;65(3):455-64.
51. **JM Blondeau.** Fluoroquinolones: mechanism of action, classification, and development of resistance. *Survey of ophthalmology*. 2004;49:S73-S8.

52. **MB Raizman, JM Rubin, AL Graves, M Rinehart.** Tear concentrations of levofloxacin following topical administration of a single dose of 0.5% levofloxacin ophthalmic solutions in healthy volunteers. *Clinical Therapeutics*. 2002;24(9).
53. **A Gwon.** Ofloxacin vs. tobramycin for treatment of external ocular disease. *Archives of Ophthalmology*. 1992;110:1234-7.
54. **HM Leibowitz.** Antibacterial effectiveness of Cipro 0.3% ophthalmic solution in the treatment of bacterial conjunctivitis. *American Journal of Ophthalmology*. 1991;112(suppl):29S-33S.
55. **IS Yalvac, NE Basci, A Bozkurt, S Duman.** Penetration of topically applied ciprofloxacin and ofloxacin into the aqueous humor and vitreous. *Journal of Cataract Refractive Surgery*. 2003;29:487-91.
56. **LC Green, MC Callegan, LS Engel, Y Shimomura, DW Jasheway, RJ O'Callaghan, JM Hill.** Pharmacokinetics of topically applied ciprofloxacin in rabbit tear. *Japanese Journal of Ophthalmology*. 1996;40:123-6.
57. **JC Wang, AS Lynch.** Transcription and DNA supercoiling. *Current Opinion in Genetics and Development*. 1993;3:764-8.
58. **JI Kato, H Suzuki, H Ikeda.** Purification and characterization of DNA topoisomerase IV in *Escherichia coli*. *Journal of Biological Chemistry*. 1993;267:25676-84.
59. **TD Gootz, KE Brightly.** Chemistry and mechanism of action of the quinolone antibacterials. In: Andriole VT, ed. *The quinolones*. London: Academic Press Limited; 1998:29-80.
60. **VE Anderson, TD Gootz, N Osheroff.** Topoisomerase IV catalysis and the mechanism of quinolone action. *Journal of Biological Chemistry*. 1998;273(28):17879-85.
61. **LM Fisher, KA Gould, XS Pan, S Patel, VJ Heaton.** Analysis of dual active fluoroquinolones in *Streptococcus pneumoniae*. *Journal of Antimicrobial Chemotherapy*. 2003;52:312-6.
62. **DC Hooper.** Mechanisms of fluoroquinolone resistance. *Drug Resistance Updates*. 1999;2:38-55.
63. **H Yoshida, M Bogaki, M Nakamura, S Nakamura.** Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. 1990;34:1271-2.
64. **EY Ng, M Trucksis, DC Hooper.** Quinolone resistance mutations in topoisomerase IV: relationship of the *flqA* locus and genetic evidence that topoisomerase IV is the primary target and DNA gyrase the secondary target of fluoroquinolones in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 1996;40:1881-8.
65. **JM Blondeau, G Hansen, K Metzler, P Hedlin.** The role of PK/PD parameters to avoid selection and increase of resistance: mutant prevention concentration. *Journal of Chemotherapy*. 2004;16(suppl):1S-19S.
66. **MW Scheld.** Maintaining fluoroquinolone class efficacy: review of influencing factors. *Emerging Infectious Diseases*. 2003;9(1):1-9.
67. **LR Peterson.** Quinolone molecular structure-activity relationships: what have we learned about improving antibacterial activity. *Clinical Infectious Diseases*. 2001;33:180S-6S.
68. **W Craig.** Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. *Clinical Infectious Diseases*. 1998;26:1-10.
69. **W Craig.** Does The Dose Matter. *Clinical Infectious Diseases*. 2001;33:S233-7.
70. **D Burgess.** Pharmacodynamic principles of antimicrobial therapy in the prevention of resistance. *Chest*. 1999:19S-23.
71. **JK Thomas, A Forrest, SM Bhavnani, JM Hyatt, A Cheng, CH Ballow, JJ Schentag.** Pharmacodynamic evaluation of factors associated the development of bacterial resistance in acutely ill patients during therapy. *Antimicrobial Agents and Chemotherapy*. 1998;42(521-7).
72. **HM Leibowitz.** Clinical evaluation of ciprofloxacin 0.3% ophthalmic solution for treatment of bacterial keratitis. *American Journal of Ophthalmology*. 1991;112(suppl):34S-47S.
73. **GT Hansen.** The mutant-prevention concentration (MPC): ideas for restricting the development of fluoroquinolone resistance. University of Saskatchewan; 2005.
74. **E Bingen, N Lambert-Zechovsky, P Mariani-Kurkdjian, C Doit, Y Aujard, F Fournerie, H Mathieu.** Bacterial counts in cerebrospinal fluid of children with meningitis. *European Journal of Clinical Microbiology and Infectious Diseases*. 1990;9:278-81.
75. **X Zhao, K Drlica.** Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. *Clinical Infectious Diseases*. 2001;33:S147-S56.

76. **K Drlica.** The mutant selection window and antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*. 2003;52:11-7.
77. **K Drlica, M Malik.** Fluoroquinolones: action and resistance. *Current Topics in Medicinal Chemistry*. 2003;3:249-82.
78. **B Sutton.** IU School of Optometry Continuing Education Topical Antibiotics Update: Fluoroquinolones.: T Greene; 2005.
79. **AW Frish, JW Tripp, CD Barrett, BE Pidgeon.** The specific polysaacharide content of pneumonic lungs. *Journal of Experimental Medicine*. 1942;76:505 - 10.
80. **Clinical and Laboratory Standards Institute (CLSI).** Table 3A Acceptable Quality Control Limits of Minimum Concentrations (µg/ml) for Fastidious Organisms. Vol. 21; 2004:110-1.
81. **JM Blondeau, X Zhao, G Hansen, K Drlica.** Mutant prevention concentrations of fluoroquinolones for clinical isolates of *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy*. 2001;45:433-8.
82. **K Metzler, G Hansen, P Hedlin, E Harding, K Drlica, JM Blondeau.** Comparison of minimal inhibitory and mutant prevention drug concentrations of 4 fluoroquinolones against clinical isolates of methicillin-susceptible and -resistant *Staphylococcus aureus*. *International Journal of Antimicrobial Agents*. 2004;24:161-7.
83. **FF Bodor, CD Marchant, PA Shurin, SJ Barankamp.** Bacterial etiology of conjunctivitis-otitis media syndrome. *Pediatrics*. 1985;76:26-8.
84. **AR Anand, KL Therese, HN Madhavan.** Spectrum of aetiological agents of postoperative endophthalmitis an antibiotic susceptibilities of bacterial isolates. *Indian Journal of Ophthalmology*. 1999;48:123 - 8.
85. **MH Goldstein, RP Kowalski, Gordon J.** Emerging fluoroquinolone resistance in bacterial keratitis: a 5-year review. *Ophthalmology*. 1999;106:1313-8.
86. **RL White, KA Enzweiler, LV Friedrich, D Wagner, D Hoban, Bosso J.** Comparative activity of gatifloxacin and other antibiotics against 4009 clinical isolates of *Streptococcus pneumoniae* in the Uinited States during 1999-2000. *Diagnostic Microbiology and Infectious Disease*. 2002;43:207 - 17.
87. **I Morrissey, R Burnett, L Viljoen, Robbins M.** Surveillance of the susceptibility of ocular bacterial pathogens to the fluoroquinolones gatifloxacin and other antimicrobials in Europe during 2001/2002. *Journal of Infection*. 2004;49:109 - 14.
88. **JM Blondeau, GT Hansen, KL Metzler, S Borsos, J Chau.** Optimal killing of *Streptococcus pneumoniae* by gemifloxacin, levofloxacin and moxifloxacin. *Novel Perspectives in Antimicrobial Action*. 2002:15 - 26.
89. **KH Ibrahim, LB Hovde, G Ross, B Gunderson, DH Wright, JC Rotschafer.** Microbiologic effectiveness of time- or concentration-based dosing strategies in *Streptococcus pneumoniae*. *Diagnostic Microbiology and Infectious Disease*. 2002;44:265 - 71.

7.0 APPENDIX A

7.1 Solutions and Media

Hemin
Dissolve 0.05 g in 1 ml of 1N NaOH. Add to 10 ml of sterile deionozed water. Final stock concentration is 5 mg/ml.

HTM (agar/broth)
Add 38 g of Mueller Hinton II agar (for broth add 38 g MHB instead), 5 g of yeast extract and 3 ml of 5 mg/ml Hemin to 1 L of distilled water. Autoclave and when cooled to 50°C add 3 ml of 15 μg/ml NAD.

NAD
Dissolve 0.05 gm in 10 ml of distilled water. Sterilize by filtering through a 0.45 millipore filter.

MHB
Add 21 g to 1 L of distilled water and then autoclave

Skim Milk
Add 200 g to 1 L of distilled water and then autoclave.

THB
Add 30 g to 1 L of distilled water and then autoclave.

TSA
Add 40 g to 1 L of distilled water and then autoclave.

8.0 APENDIX B

8.1 Preparation of Antimicrobial Agents

Ciprofloxacin
Dissolve 0.1 g into 10 ml of sterile distilled water. At 84.6% purity the final concentration is 8460 μg/ml.

Gatifloxacin
Dissolve 0.035 g into 10 ml of sterile distilled water. At 93.3% purity, the final concentration is 3265.5 μg/ml.

Levofloxacin
This agent is available in an injectable form at 25 mg/ml. Dilute to desire concentration.

Moxifloxacin
Add 0.02 g to 10 ml of sterile distilled water. At 87.8% purity the final concentration is 1756 μg/ml.

Ofloxacin
Add 0.05 g to 10 ml of sterile distilled water. Add 4 drops of NaOH to help dissolve the powder. The final concentration is 5000 μg/ml.

9.0 APPENDIX C

10.1 Suppliers

10.1.1 Media

Chocolate Agar Plates	PML Microbiologicals, Winnipeg, MB
Haemophilus Test Media	PML Microbiologicals, Winnipeg, MB
Mueller Hinton Broth (MHB)	Becton, Dickinson and Co., Sparks, MD
Todd Hewitt Broth (THB)	Becton, Dickinson and Co., Sparks, MD
Tryptic Soy Agar (TSA)	Becton, Dickinson and Co., Sparks, MD
5% Sheep Blood	Oxoid
Yeast Extract	Becton, Dickinson and Co., Sparks, MD

9.1.1 Antimicrobial Agents

Ciprofloxacin	Bayer, West Haven, CT
Gatifloxacin	Allergan Inc., Irvine, CA
Levofloxacin	Janssen-Ortho Inc., North York, ON
Moxifloxacin	Bayer, Mt. Prospect, IL
Ofloxacin	Allergan Inc., Irvine, CA

9.1.2 Reagents and Chemicals

95% Alcohol	Commercial Alcohols Inc., Brampton, ON
Glycerol	BDH Inc., Toronto, ON
Hemin	SIGMA
β-Nicotinamide Adenine Dinucleotide	SIGMA,
Saline	Baxter, Deerfield, IL
Skim Milk	Becton, Dickenson and Co., Sparks, MD
Sodium Hydroxide (NaOH)	BDH Inc., Toronto, ON

9.1.3 Disposable Labware

200 µl Pipette Tips	VWR International, Edmonton, AB
Corning Cryovials	Corning Inc., Corning, NY
Cuvettes	Fisher Scientific, USA
Glass Tubes	Fisher Scientific, USA
Latex Gloves	Fisher Scientific, USA
McFarland Tubes	Fisher Scientific, USA
Microtitre Plates	Sarstedt, Newton, NC
Pasteur Pipettes	Fisher Scientific, USA
Sterile Plastic Petri Plates	Fisher Scientific, USA
Swabs	Fisher Scientific, USA
Wooden Applicator Sticks	Puritan, Guilford, Maine

9.1.4 Equipment

20 µl, 200 µl and 1 ml Pipettors	Gilson Company, Inc., Lewis Cener, OH
-70°C Freezer	Forma Scientific Inc., Marjetta, OH
Avanti J-E Centrifuge	Beckman Coulter, Palo Alto, CA
Colorimeter	Hach Company, Loveland, CO
Hot Plate/Stirrer-Model 300T	Fisher, Scientific, USA
pH Meter	Corning Inc., Corning, NY
Oxygen Incubator	Hotpack Corp., Philadelphia, PA
Shaking Water Bath	Mandel Scientific Co., Guelph, ON
Spectrophotometer	Pharmacia, Cambridge, England
Vortex (Mini-shaker Model 58)	Fisher Scientific, USA
Weigh Scale – Mettler PC440	DeltaRange, Zurich, Switzerland